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OPPOSING ROLES OF THE μ-OPIOID AND NOCICEPTIN/ ORPHANIN FQ RECEPTORS IN OLIGODENDROCYTE DEVELOPMENT AND MYELINATION

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A thesis submitted in partial fulfillment of the requirements for the degree of Master of Science at Virginia Commonwealth University

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"It's good to be curious about many things." Fred Rogers

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LIST OF ABBREVIATIONS

cAMP – cyclic adenosine monophosphate

cdk5 – cyclin-dependent kinase 5

CDM – chemically defined medium

CMT - Charcot-Marie-Tooth disease

CNP - 2', 3'-cyclic nucleotide 3'-phosphodiesterase

CNS – central nervous system

CTOP – D-Phe-Cys-Tyr-D-Trp-Orn-Thr-PenThr-NH2 [Disulfide Bridge: 2–7])

DAMGO – [D-Ala^2, N-MePhe^4, Gly-ol]-enkephalin

DMEM-F12 – Dulbecco's Modified Eagle Medium: Nutrient Mixture F-12

DNAse – deoxyribonuclease

DOR - δ-opioid receptor

EAE - experimental autoimmune encephalomyelitis

ERK - extracellular-signal-regulated kinases

GPCR – G-protein coupled receptor

HBBS – Hank's Balanced Salt Solution

HEPES – 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid

J-113397 – [(3R,4R)-1-cyclooctylmethyl-3- hydroxymethyl-4-piperidyl]-3-ethyl-1, 3-dihydro-2H-benzimidazol-2-one

KOR - κ-opioid receptor

MAG – myelin-associated glycoprotein

MAPK - mitogen-activated protein (MAP) kinase

MBP – myelin basic protein

MEK - mitogen-activated protein (MAP) kinase kinase

MOG - myelin oligodendrocyte glycoprotein

MOR - μ-opioid receptor

MS - multiple sclerosis

NOPR – nociceptin/orphanin FQ receptor

OGF – opioid growth factor

OLGs – oligodendrocytes

PAGE – polyacrylamide gel electrophoresis

PBS – phosphate buffered saline

PKA – protein kinase A

PLP – proteolipid protein

PNS – peripheral nervous system

SDS – sodium dodecyl sulfate

SEM – standard error of the mean

ABSTRACT

OPPOSING ROLES OF THE μ-OPIOID AND NOCICEPTIN/ORPHANIN FQ RECEPTORS IN OLIGODENDROCYTE DEVELOPMENT AND MYELINATION

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While the classical function of myelin is to facilitate saltatory conduction, this membrane and the myelin-making oligodendrocytes (OLGs) are now recognized as regulators of plasticity and remodeling in the central nervous system (CNS). Thus, OLG maturation and myelination are highly vulnerable processes along CNS development. We previously showed that rat brain myelination is altered by perinatal exposure to buprenorphine, an opioid analogue in clinical trials for the treatment of pregnant opioid addicts. We now found that the *in vivo* effects on myelination could result from direct alteration in the balance between μ-opioid receptor (MOR) and nociceptin/orphanin FQ receptor (NOPR) activities in the OLGs. Furthermore, we found that myelination could also be affected by the FDA-approved methadone. A delicate balance between MOR and NOPR signaling may play a crucial role timing OLG maturation and myelin formation and exogenous opioids may disrupt this interplay, altering the developmental pattern of brain myelination.

INTRODUCTION

Epidemiological surveys show that opioids, including morphine, heroin and codeine, are among the most frequently abused drugs (Cicero et al., 2005, Compton and Volkow, 2006). Furthermore, studies in the United States showed that this trend is even observed within the pregnant population (Bolnick and Rayburn, 2003). In addition, it is a currently accepted medical practice to prescribe women with opioids to ameliorate pain during pregnancy and as such, it is critical to fully understand the potential effects that these exogenous opioids could have on the developing brain of the fetus. The placenta serves to connect the circulatory systems of the mother and child, allowing for the transfer of gases and nutrients. However, the traditional assumption that exogenous molecules would not pass from the mother to the developing baby has been shown to be incorrect as many drugs are now known to cross the placenta by both active and passive transport mechanisms (Syme et al., 2004). Studies of infants exposed to exogenous opioids *in utero* report reduced head circumference, decreased attention, altered fine motor coordination and a greater risk for Sudden Infant Death Syndrome (Cohen et al., 1982, Johnson and Rosen, 1982, Rosen and Johnson, 1982, Kandall et al., 1993, Jansson et al., 2011a).

In addition to treatment with opioids for their anti-nociceptive properties, synthetic opioids, such as buprenorphine and methadone, are currently used as therapy for pregnant opioid addicts with the goal of reducing the amount of "street drug" use and ensuring a safer pregnancy for the mother and child. Clinical trials with buprenorphine treatment have shown a reduction in neonatal abstinence syndrome (Jaffe and O'Keeffe, 2003, Amass et al., 2004).

Treatment with methadone has been less successful clinically, with up to 80% of the prescribed pregnant patients continuing to use illicit drugs in addition to methadone. However, it has also been reported that treatment with methadone was shown to reduce heroin exposure for the fetus and eliminate risks of fetal withdrawal (McCarthy, 2012).

Importantly, the effects of buprenorphine or methadone exposure *in utero* on brain development have not been thoroughly studied. These issues are of great clinical significance because oligodendrocytes (OLGs), the myelinating cells of the central nervous system (CNS), express multiple opioid receptors (Knapp et al., 1998), to which buprenorphine and methadone can bind. Buprenorphine, a semi-synthetic derivative of either thebaine or oripavine, is a known μ-opioid receptor (MOR) partial agonist and a kappa opioid receptor (KOR) antagonist and, as our results show, also a nociceptin receptor (NOPR) agonist. On the other hand, methadone is known to be a long-acting MOR agonist. These, and other exogenous opioids, could bind to these receptors and, as this thesis will describe, significantly alter OLG development and differentiation, possibly affecting brain development.

This laboratory has previously demonstrated that in animals perinatally exposed to buprenorphine, the caliber of the myelinated axons was increased, with a disproportionally thinner myelin sheath (Sanchez et al., 2008). Furthermore, buprenorphine altered the temporal pattern of brain myelination. The expression of myelin proteins in the brain of the pups was accelerated by exposure to therapeutic doses of buprenorphine while delayed by the higher supra-therapeutic doses of the drug. Myelin, in addition to its traditional role as an insulator of axons, has been shown to work with other factors to control axonal extension and radial growth

and establish the nodal and paranodal structures and ion channel localization at the nodes of Ranvier. Therefore, it is important to understand how exogenous opioids could affect the synthesis of myelin and its ability to properly perform its functions in the CNS. This thesis examines the interplay between MOR and NOPR and the effects of these receptors on OLG differentiation and myelination.

MYELIN

In 1858, the German pathologist Rudolf Virchow first used the Greek word "myelo" (meaning marrow) to term "myelin" and describe "the particular structure" that surrounded some of the nerve fibers in the body (Hartline, 2008). A short two decades later, the French anatomist Louis-Antoine Ranvier correctly hypothesized that the fatty sheath was involved in insulation (Ritchie, 1984). However, this membrane was described as early as 1839 by Theodor Schwann, who believed that the "white substance" may be the cell membrane of the nerve or a secondary nerve cell (Münzer, 1939).

Structure

The myelin membrane, found mostly in jawed vertebrates (the subfamily Gnathostomata), is a complex and specialized membrane that wraps around the axons of nerves in both the CNS and peripheral nervous system (PNS). This multilayered and highly polarized membrane is produced by OLGs in the CNS and by Schwann cells in the PNS, and as further described in the following sections, myelin functions are now known to expand beyond that of the classical role as an axonal insulator responsible for the rapid "saltatory" conduction of impulses.

Myelin can be visualized by electron microscopy as concentrically alternating layers of electron-dense and light lines (**Figure 1)**. The electron-dense line or "major dense line" corresponds to the closely condensed cytoplasmic sides of the myelinating membrane, while the light or "intraperiod line" results from the apposition of the extracellular surface of adjacent membrane wraps (Baumann and Pham-Dinh, 2001). Thus, these membranes are tightly compacted around each individual, myelinated axon and generate multiple successive myelinated segments or "myelin internodes" averaging 150 μm in length along the axon. The myelin internodes are segregated by the nodes of Ranvier, naked regions of the axons that are characterized by a high density of sodium channels (120,000/μm^2) and where the generation of action potentials occurs resulting in "saltatory" nerve conduction (**Figure 2**). The lateral margins of the individual membrane wraps contain regions of "uncompacted" myelin. These paranodal loops, surrounding the node of Ranvier, may also serve to restrict lateral diffusion of adjacent proteins of the axolemmal membrane (Rosenbluth, 1976).

In the PNS, each Schwann cell will wrap a single discrete axon producing a single myelin internode, a process which is drastically different from that of myelination in the CNS, in which an individual OLG can myelinate up to 50 different axons (Pfeiffer et al., 1993). Therefore, in the CNS, either cell death or defective differentiation of a few OLGs can both result in widespread lack of myelin, affecting a greater amount of axons than in the PNS. However, the myelin membrane has also been shown to be surprisingly stable, remaining over a period of weeks even after ablation of the OLGs (Aggarwal et al., 2011).

Figure 1: Cross section of myelin showing the characteristic presence of alternating electron dense and light lines. Mature OLGs extend their processes generating many wraps around the axon. An electron microscope image of a myelinated axon viewed in cross section displays the periodicity of electron dense and light lines. It is proposed that MBP fuses the cytoplasmic side of the membranes to form the electron dense line. The tight apposition of the outer face of the membranes may be mediated by PLP. (Soldan and Pirko, 2012)

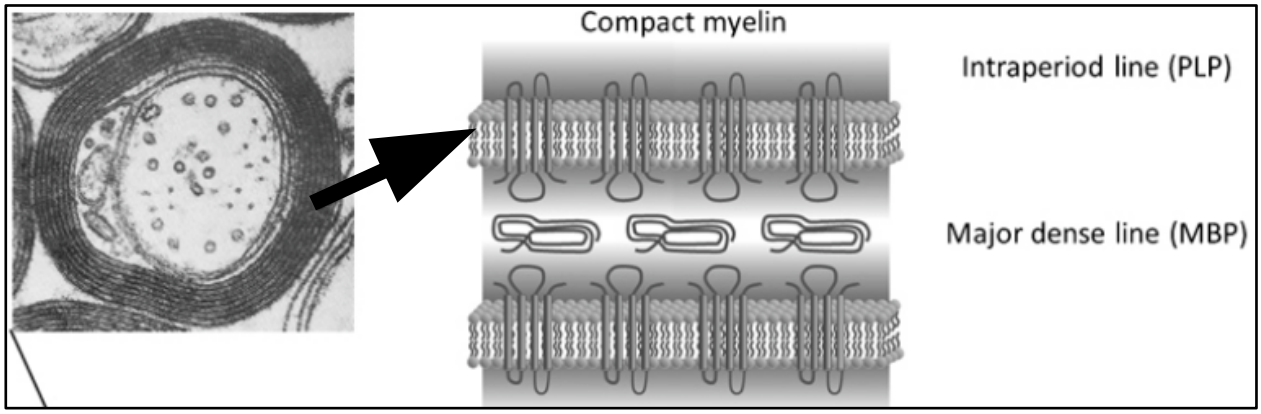
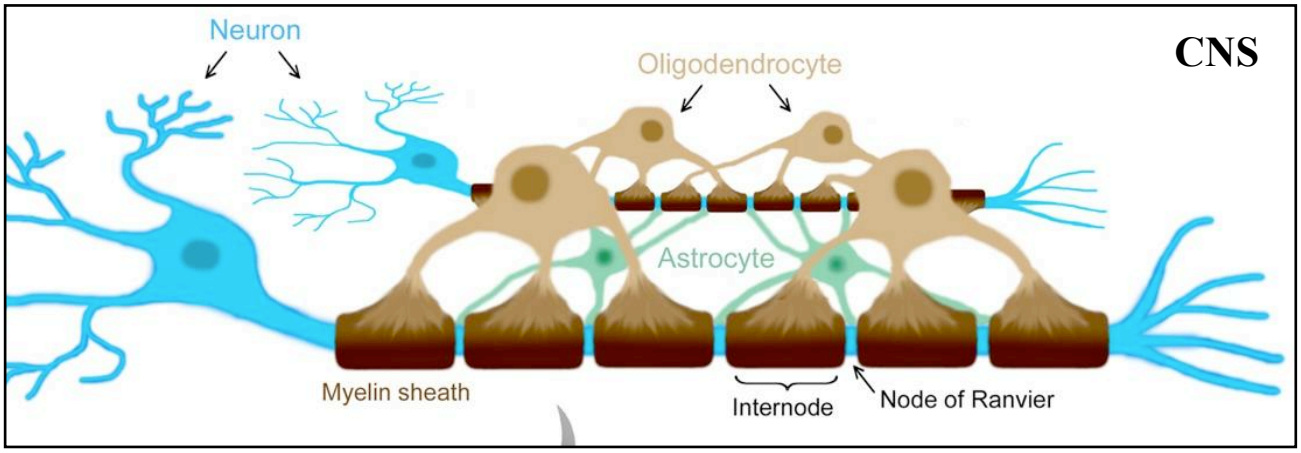

Compact myelin
Intraperiod line (PLP)
Major dense line (MBP)

Figure 2: **Cellular associations of the myelin membrane in the CNS.** The myelin membrane of CNS is produced by OLGs and serves to increase the speed at which the action potential propagates along the axon due to the high fat content, which works as an insulator. (Jackman et al., 2009) Perinodal astrocytes may function in the aggregation of sodium channels at the node of Ranvier (Black et al., 1989). Modified from Jackman et al., 2009.


Neuron
Oligodendrocyte
CNS
Astrocyte
Myelin sheath
Internode
Node of Ranvier

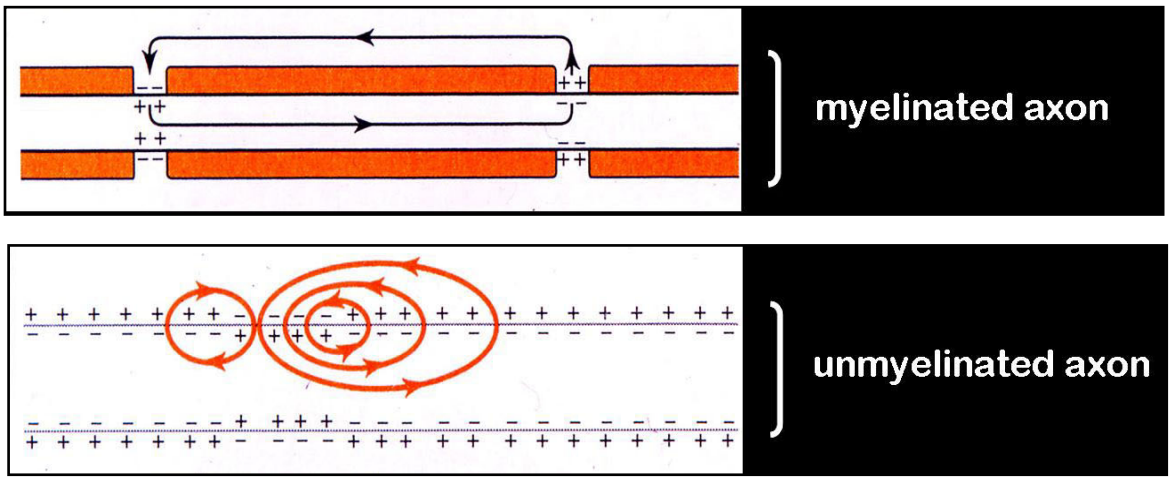
Function

The classical, well-understood physiological role of the myelin sheath is that of a facilitator of saltatory conduction along the axon, allowing nerve impulses to propagate more effectively than they could along a similarly sized, unmyelinated axon (**Figure 3**). As mentioned above, this is achieved by both insulating the axons with myelin and by restricting the localization of sodium channels to the nodes of Ranvier. The restriction of the sodium channels, and therefore the generation of action potentials, to these nodes allows for the signal to "jump" or "dance" from node to node, decreasing the loss of signal and increasing the speed at which the signal moves along the axon. In an unmyelinated axon, action potentials are still propagated in forward direction along the axon due to the absolute refractory period, albeit at a slower rate of transmission, averaging 1 m/s compared to a myelinated axon speed of transmission of 100 m/s. Organisms lacking myelination can increase the speed of conduction by decreasing the interior resistance, through an increase in fiber diameter. Example of this are the large sensory axons of some copepods, such as the giant squid (Hartline and Colman, 2007).

In addition to its traditional role as axonal insulator, the myelin membrane has also been shown to play a crucial function in the control of axonal extension and radial growth and the establishment the nodal and paranodal structures and ion channel localization at the nodes of Ranvier (Dupree et al., 2004, Marcus et al., 2006, Rasband, 2006). The myelin membrane also provides trophic support to the underlying axons and maintains their integrity. However, it is important to note that both myelin and the myelin-making cells have important complementary roles. Studies in myelin-deficient mutant mice showed that the OLGs themselves can also induce neurofilament accumulation and therefore, promote axonal radial growth (Sanchez et al.,

1996); and while myelin is required for the long-term maintenance of sodium-channel domains, OLGs can also contribute to this function in a myelin-independent manner (Dupree et al., 2004). In light of these wide-ranging functions of myelin and the OLGs, it is important to fully understand diseases or drugs that may affect this membrane or the myelinating glial cells.

Figure 3: **Propagation of nerve impulses along myelinated and unmyelinated axons.** Conduction of nerve impulses in an unmyelinated axon occurs by continuous membrane depolarization mediated by ion channels located along the entire length of the axon. In contrast, the myelin membrane allows for rapid, saltatory conduction along the myelinated axon, with the signal jumping from node to node. Myelin internodes inhibit the loss of charge through the membrane, restricting depolarization to the nodes of Ranvier. Modified from Basic Neurochemistry, edited by Siegel, Albers, Brady and Price, Elsevier, 2006.


myelinated axon
unmyelinated axon

Myelin Composition

The myelin membrane is composed of roughly twice the amount of lipids as compared to proteins. Typical cellular membranes on the other hand are made up of mostly proteins, so the myelin membrane is unique in this regard. This high lipid concentration is responsible for the insulating properties of myelin that as indicated above, contribute to the saltatory conduction of electrical impulses. Major myelin lipids include cholesterol, phospholipids and in particular cerebrosides and sulfatides, two galactolipids that appear to play important roles in myelin function and structural organization (Dupree et al., 1998, Marcus et al., 2002, Marcus et al., 2006, Fewou et al., 2010). The proteins in myelin are largely unique to this membrane and to Schwann cells and OLGs (Campagnoni and Macklin, 1988) and they are not expressed by other cell types (Marcus et al., 2006). The majority of the myelin's protein weight is comprised of approximately 30% myelin basic protein (MBP) and 50% proteolipid protein (PLP), with 2', 3'-cyclic nucleotide 3'-phosphodiesterase (CNP) and myelin-associated glycoprotein (MAG) making up 4% and 1%, respectively. Another myelin protein component is the myelin oligodendrocyte glycoprotein (MOG), a transmembrane protein in CNS myelin that has been implicated as a main auto-antigen in multiple sclerosis (MS) (Iglesias et al., 2001).

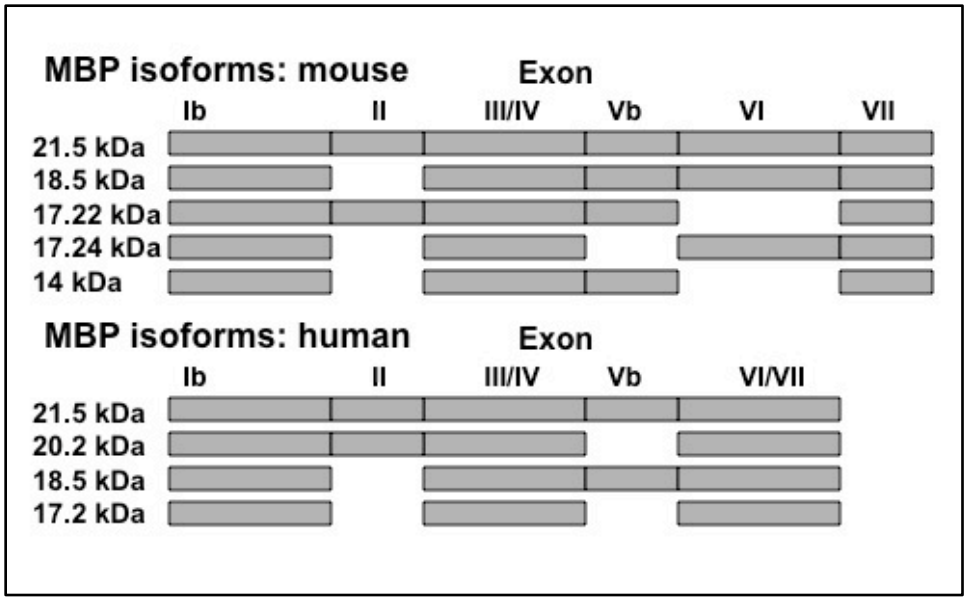
The next section will focus on the MBPs as studies in this thesis used MBP expression as a marker of OLG maturation.

Myelin Basic Proteins

The MBPs are actually part of a large family of proteins, containing a variety of isoforms, due to the existence of three transcription start sites and alternative splicing of the

Golli (genes of oligodendrocyte lineage)-MBP gene complex (de Ferra et al., 1985, Takahashi et al., 1985, Kamholz et al., 1986, Newman et al., 1987, Aruga et al., 1991) (**Figure 4**). Isolation of these proteins from the myelin membrane allowed for the initial sequencing of MBP in 1971 (Eylar et al., 1971). The proteins in this family have both different molecular mass and expression profiles (Carnegie, 1971). The Golli-MBP gene is approximately 105 kb in the mouse and 108 kb in humans (Campagnoni et al., 1993, Pribyl, 1993) and contains three transcription start sites and at least 11 exons. The gene encodes not only MBPs but also Golli proteins that are expressed in both glial cells and the immune system. The major classic isoforms of MBP are generated by initiation of transcription at the third start site of the Golli-MBP gene and are 21.5, 20.2, 18.5 and 17.2 kDa in humans and 21.5, 20.2, 18.5, 17.24, 17.22 and 14kDa in rodents (Barbarese et al., 1978, de Ferra et al., 1985, Kamholz et al., 1986, Harauz et al., 2004). All isoforms contain domains encoded by exons I, III, IV and VI while only the 21.5 and 17.22 kDa forms contain exon II encoded regions. Interestingly, exon II-containing isoforms are expressed at higher proportion at early developmental stages while the exon II-lacking species are considered to be the major MBPs expressed later in OLG maturation and myelination (Barbarese et al., 1978, de Vries et al., 1997, Pedraza, 1997). Furthermore, the various MBPs also exhibit different intracellular localizations with exon II-containing isoforms expressed in the plasma membrane and myelin and exon II-lacking isoforms majorly localized in the cytoplasm and nucleus (Staugaitis, 1990, Huang et al., 1991). In the human, the 20.2 and 21.5 kDa isoforms appear earlier in development and are re-expressed during periods of re-myelination, as it occurs with chronic Multiple Sclerosis (MS) lesions (Capello et al., 1997).

Figure 4: Alternative splicing of the Golli-MBP gene results in a wide variety of isoforms in rodents and humans. MBP isoforms are both developmentally and spatially regulated with a variety of splicing variants in rodents and humans. Modified from (Boggs, 2006).


MBP isoforms: mouse
Exon
Ib
II
III/IV
Vb
VI
VII
21.5 kDa
18.5 kDa
17.22 kDa
17.24 kDa
14 kDa
MBP isoforms: human
Exon
Ib
II
III/IV
Vb
VI/VII
21.5 kDa
20.2 kDa
18.5 kDa
17.2 kDa

Post-translational modifications of MBPs include phosphorylation, deamidation, methylation, deimination and N-terminal acylation (Kim et al., 2003). MBPs appear to play a role in compaction of the CNS myelin, as described by studies in the shiverer mouse, in which the electron-dense line is lacking (Privat et al., 1979). These mice display a tremor early in life, which becomes more severe with age, and have a greatly shortened lifespan due to the effects of the large deletion of a portion of the MBP gene.

Recent studies suggest that MBP could also play a role in signal transduction events as the classical 18.5 and 21.5 kDa splicing isoforms were shown to inhibit calcium influx by OLGs by a process that involves regulation of voltage-gated calcium channels (Smith et al., 2011). Thus, it is possible that different intracellular localization as well as developmental expression of the MBPs is a reflection of the variety of roles that these proteins may play in OLG maturation and myelination.

Myelin Associated Disease States

MS is likely the most well known among all diseases affecting the process of myelination or the integrity of the myelin membrane. This is a chronic, inflammatory, autoimmune disease of the CNS, first described by Jean-Martin Charcot in 1868 (Compston, 1992) and it is caused by damage to the myelin sheath after development. The name "multiple sclerosis" is a reference to the "scars" or lesions in the nervous system. Worldwide cases of MS are estimated to be between 1.1 million and 2.5 million and this disease is the leading cause of disability in the young adult population of the western world (Noseworthy et al., 2000). MS is currently thought to involve an attack by the immune system on the nervous system, for reasons

not yet fully understood. However, recent studies also showed loss of OLGs and neurons at the initial stages of the disease that may not be associated with the infiltrating inflammatory cells. Adding in complexity to the problem of MS, is the observation that this disease can vary widely in severity, clinical progression and age of onset. The common symptoms at the onset of MS may include impaired vision due to damage of the optic nerve and problems with sensation (Baranzini, 2009). The severity of the lesion may vary from having demyelination with little OLG damage to involving more widespread tissue damage with myelin loss accompanied by apoptosis of OLGs and axonal degeneration.

Among other diseases that result in myelin membrane damage are also Guillain-Barré and Charcot-Marie-Tooth disease (CMT). Guillain-Barré is a disorder triggered by abnormal response to antigens, including those in flu vaccines, that result in autoimmune attack of the PNS myelin (Lehmann et al., 2009). CMT is the result of inherited disorders of the PNS, such as a duplication of the PMP22 gene, which is observed in the most common form of the disease. This gene encodes PMP22 which is a major protein component of myelin in the PNS (D'Urso et al., 1999). As with MS, individuals affected by these demyelinating diseases present symptoms that may include problems with vision, hearing and movement.

In addition, more recent studies uncovered a growing number of neurological diseases and psychotic disorders associated to myelin pathology during adolescence, including among others, schizophrenia (Chambers and Perrone-Bizzozero, 2004, Kubicki et al., 2005, Walterfang et al., 2005, Kerns et al., 2010, Whitford et al., 2012), bipolar disorder (Chambers and Perrone-Bizzozero, 2004, Brambilla et al., 2009, Mahon et al., 2010) and autism (O'Hearn et al., 2008).

These later findings are particularly important because they point to a crucial role of myelin in CNS plasticity and development.

Such a large variety of disorders involving the synthesis and integrity of myelin underscores the importance of better understanding the complex biology of the myelinating glial cells.

OLIGODENDROCYTES

In 1846, Virchow described the "nervenkitt" (nerve glue, i.e. neuroglia) of the brain, which he originally hypothesized was connective tissue (Kettenmann and Verkhratsky, 2008). These glial cells, which we now know, include OLGs, astrocytes and microglia, surprisingly make up the majority of cells in the nervous system. Moreover, the percentage of glial cells in the nervous system increases in general with evolution, representing up to 25% of the cells in *Drosophila* and, depending on the regions, 50-70% of the cells in the primate and human brain (Pfrieger and Barres, 1995, Azevedo et al., 2009, Garcia-Amado and Prensa, 2012). This is particularly true in the white matter of the CNS, where glia represents the great majority of the cells, as one would expect due to the myelination. However, it took many years beyond Virchow's initial observations until the existence and identity of the different glial cell types was fully appreciated. It was not until the 1930's that, using silver carbonate impregnation, del Río-Hortega furthered the work of Ramón y Cajal and discovered the "interfascicular glia" (OLGs) and microglia (McGeer and McGeer, 2011). del Río-Hortega was also the first to term these interfascicular glial cells "oligodendrocytes" due to the processes stained by the metallic impregnation techniques. In working with del Río-Hortega, Penfield hypothesized in 1924 that

these cells may play a role in the elaboration and maintenance of myelin, similar to what was already known at that time about of the function of the Schwann cells (Penfield, 1924). This early work and the discovery of glial cells opened an exciting new era in research and the awareness that knowledge on the generation and function of these cells is crucial to the understanding of the CNS, both in health and disease.

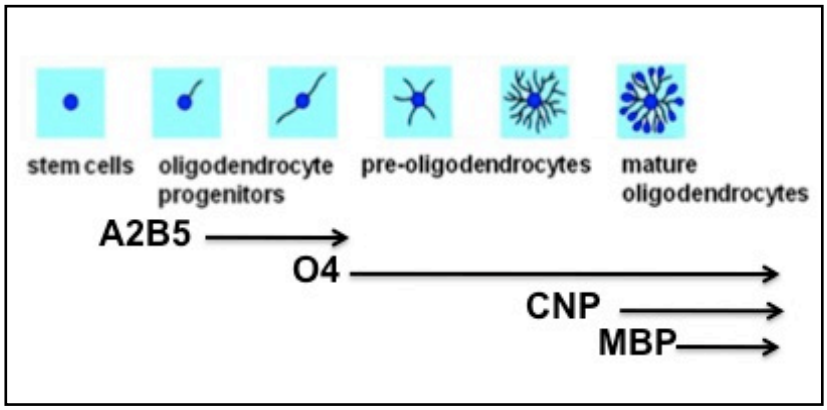
OLG Differentiation and Myelination

OLGs must reach their fully differentiated state prior to production of the myelin membrane and support of the axon. Therefore, it is necessary to understand what are the endogenous signals that drive the maturation of these cells and, relevant to this thesis, how exogenous molecules and conditions could affect this process. However, the molecular mechanisms that control OLG differentiation are not fully understood. OLGs originate from the neural tube as proliferative, migratory O-2A precursor cells, which can be labeled by A2B5 (**Figure 5**), a monoclonal antibody that detects gangliosides (Dubois et al., 1990). These immature cells have a simple bipolar shape and may have one or two processes protruding from the cell body. As they continue to differentiate, these progenitors become multipolar, lose their ability to be recognized by the A2B5 antibody and are instead, pre-OLGs that can be labeled with the O4 monoclonal antibody (Sommer and Schachner, 1981). At least in culture, this stage of differentiation is induced by the presence of thyroid hormone (Almazan et al., 1985). As terminal differentiation begins, immature OLGs first synthesize the myelin enzyme CNP and galactocerebrosides that are recognized by the O1 monoclonal antibody (Sommer and Schachner, 1981). As the cells finally become mature OLGs capable of myelination, they

acquire a complex multi-branched morphology and they can additionally be identified by the expression of MBP, PLP, MAG and MOG (Zurbriggen et al., 1984, Dubois-Dalcq et al., 1986).

When cultured in chemically defined medium, OLG progenitors are able to both proliferate and later differentiate into post-mitotic OLGs that express myelin-specific proteins even in the absence of neurons. Therefore, the ability of the progenitor cells to mature into OLGs seems to be intrinsically programmed. However, it has also been shown that co-culture of OLGs with neurons results in a four-fold increase in the expression of MBP and PLP mRNA levels and the stimulatory signals responsible for this effect appear to depend on axo-glial interactions (Macklin et al., 1986, Bozzali and Wrabetz, 2004).

Figure 5: Selected markers along OLG differentiation. Proliferative, migratory O-2A precursor cells are recognized by the A2B5 antibody. The O4 antibody reacts with the sulfated glycolipid antigen, Proligodendrocyte Antigen, on late OLG progenitors and with sulfated galactosylcerebroside on pre-OLGs and OLGs that have undergone terminal differentiation. Immature OLGs, which have begun terminal differentiation, are identified by the additional synthesis of CNP. On the other hand, MBP expression is specific of the mature OLGs potentially capable of myelination.


stem cells
oligodendrocyte progenitors
pre-oligodendrocytes
mature oligodendrocytes
A2B5
O4
CNP
MBP

In vivo, MBP is expressed in OLGs that have migrated to the axons and are at the stage just prior to the ensheathment of the axon (Asou et al., 1995, Butt et al., 1997). Thus, it is also logical to hypothesize that both neuronal messages and axo-glial interactions are crucial to signal the mature OLGs to begin the enwrapping of the axons and their myelination. These signals appear to also control complex mechanisms of protein transport and localization. The mature myelinating OLG has a polarized phenotype, with the extending myelin membrane differing in both composition and structure from the plasma membrane of the OLG cell body. This polarization requires specific timing for the production of the myelin components and their transport to the forming sheath. Many of the myelin proteins are transmembrane species, synthesized in the endoplasmic reticulum and then transported to the Golgi apparatus and to the plasma membrane by vesicular transport. However, the mechanism of transport and intracellular localization appears to be specific for each of the myelin proteins. For example, in transfected kidney cells, MOG was found to be transported to the basolateral membrane, while PLP was transported to the apical membrane (Kroepfl and Gardinier, 2001). Recently, *in vitro* and *in vivo* studies determined that transport of PLP to the plasma membrane of the OLGs is a complex process that appears to involve two different trafficking systems mediated by the R-soluble N-ethylmaleimide-sensitive factor attachment proteins VAMP3 and VAMP7. While VAMP3 is important for the fusion of endosome-derived vesicles along the secretory pathway in OLGs, VAMP7 regulates the exocytosis of PLP from the late endosomal compartment (Feldmann et al., 2011).

Of the major myelin proteins, CNP is the first to be expressed by the OLGs and is transported in a monomeric state to the processes. The membrane-association of CNP requires isoprenylation (Braun et al., 1991). Additionally, CNP has been shown to bind actin filaments and microtubules, suggesting that localization of CNP may involve association with these cytoskeletal proteins (De Angelis and Braun, 1996).

The situation is quite different for MBP as the MBP mRNA is transported along the microtubules in granules together with the machinery for translation and protein synthesis close to the insertion sites (Barbarese et al., 1995), allowing for faster incorporation of the protein into the myelin structure (Colman et al., 1982). Fyn kinase phosphorylates the heterogeneous ribonucleoprotein F (hnRNP F), which results in its release from MBP mRNA, allowing translation to occur (Laursen et al., 2011, White et al., 2012). Thus, it is clear that controlling OLG development and myelination involves the concerted action of multiple systems that still remain poorly understood. The next sections are dedicated to the effects of opioids in OLGs and the process of myelination.

Opioid Receptors Present in Oligodendrocytes

OLGs express multiple opioid receptors along their differentiation, including the μ-opioid receptor (MOR) and κ-opioid receptor (KOR) (Knapp et al., 1998), to which exogenous opioids such as buprenorphine and methadone can bind. Previous results from this laboratory have shown that exposure to an exogenous opioid, such as buprenorphine or methadone, can affect the development of the CNS through alterations of the timing and quantity of myelin

produced by the OLGs (Sanchez et al., 2008), further supporting the *in vivo* role of the opioid system in myelination.

OPIOIDS

History

The early use of opium reaches back to antiquity and therefore it is difficult to assess where the poppy plant (*Papaver somniferum*), from where opium is derived, was originally grown. However, it is known that the ancient Sumerians not only cultivated the poppy plant, but also named it "hul gil" which means "plant of joy", suggesting that its use to alleviate pain was already well understood (Hamarneh, 1972). Furthermore, indication of early opioid use can be found in the Ebers Papyrus, an Egyptian document from around 1550 BC, which contains medical information with roughly 700 formulas and remedies for various illnesses and maladies. One of these remedies was to be used in a four-day treatment for crying children and was described as a preparation that resulted from mashing the grain and straining of the pulp of the poppy plant (Crocq, 2007). Traders then brought opium to China and India in the eighth century (Dwarakanath, 1965, Fort, 1965) and, by the thirteenth century, its use had spread to Europe as well.

The opium trade escalated during the 1800s, with England importing an estimated 200,000 kg of opium from China (Poroy, 1981). This epidemic of drug use led to the first Opium War in 1840, which the British won, keeping their country open to the opium trade. By 1906, nearly 27% of the adult males were opioid addicts, which, with this percentage

representing 13.5 million addicts, was likely the largest example of opioid addiction in world history (Fang et al., 2006).

In the early 1800s, the active ingredient in opium was discovered and isolated by Friedrich Sertürner and named morphine, after Morpheus, the Greek god of dreams (Brownstein, 1993). This drug was then used for surgery and chronic pain relief, however it was also widely abused. Synthetic opioids such as methadone were developed in an effort to find safe compounds with similar anti-nociceptive effects to those of morphine. Methadone has a long half-life of 15-60 hours and therefore offers a more attractive method for controlling pain as patients are more likely to follow through on medication adherence (Chou, 2009). It should be pointed out that opioids are molecules that can bind and act on the opioid receptors, while opiates are natural alkaloids from the previously mentioned poppy plant. The majority of these drugs currently used are synthetic opioids.

These drugs are available in the United States by prescription only, due to the growing problem of abuse. San Francisco was one of the first cities to enact legislation concerning the smoking of opium in 1875; before the federal government intervened with a registration for all parties involved in importing, exporting or manufacturing opium or cocaine through the Harrison Narcotics Tax Act of 1914 (Das, 1993). Physicians were exempt from this law when using these drugs in their practice or when prescribing narcotics to addicted patients at maintenance levels. However, five years later, this was changed to prevent physicians from continuing to prescribe these drugs to addicted patients who were not attempting to decrease and end their opioid use.

Endogenous Opioid System

In 1973, several groups independently described the existence of membrane receptors for opiates in the brain (Pert and Snyder, 1973, Simon et al., 1973, Terenius, 1973). Work on these receptors was furthered in the 1990s, with the characterization of the multiple endogenous opioid receptor subtypes: μ (MOR), δ (DOR) and κ (KOR) opioid receptors (Evans et al., 1992, Chen et al., 1993, Meng et al., 1993, Kieffer et al., 1994). These molecules are seven-transmembrane domain proteins that belong to the family of G-protein coupled receptors (GPCRs). These receptors follow a similar pattern of activation in which the ligand binding induces a conformational change resulting in the exchange of the previously bound GDP by GTP, the release of the β and γ subunits, and, depending on the α subunit type ($G\alpha_s$, $G\alpha_{i/o}$, $G\alpha_{q/11}$, $G\alpha_{12/13}$), different downstream signal transduction effects that can include activity changes at the level of adenylate cyclase, phospholipases, kinases or ion-channels (Forse, 2000) (**Figure 6**). In addition to its classical role in the control of nociception, the opioid system has been found to play a role in a variety of physiological functions, including reward-related behavior (Rutten et al., 2011), gastrointestinal transit (Duraffourd et al., 2012) and regulation of the immune system (Finley et al., 2008).

As this thesis describes a possible role for endogenous opioids in brain development, it is particularly interesting that in neurons, KOR activation by the endogenous peptide dynorphin results in decreased calcium currents, which may be due to inhibition of the release of glutamate (Kelamangalath et al., 2011). These transient calcium currents are thought to play a role in the regulation of neuronal plasticity (Spitzer et al., 1995) and therefore, it is possible that KOR may assist in CNS development through regulation of calcium levels. Additionally, the MOR agonist

DAMGO was found to inhibit calcium channels in isolated neurons and may also play a role in the regulation of neurotransmitter release (Rhim and Miller, 1994).

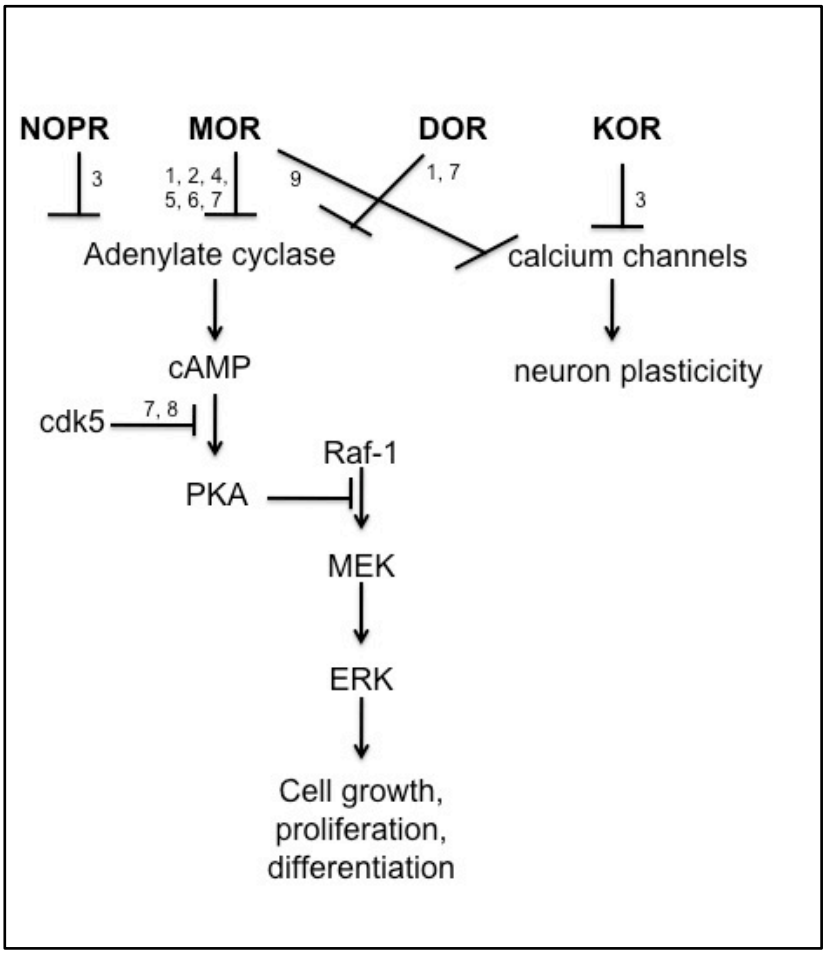
Stimulation of the MOR in both human neuroblastoma cells and in rat brain striatum membranes has been shown to couple to adenylyl cyclase (Carter and Medzihradsky, 1993). In addition, human kidney cells expressing MOR and exposed to morphine and to methadone exhibited a concentration dependent inhibition of adenylate cyclase and cyclic adenosine monophosphate (cAMP) accumulation (Lee et al., 2011). Similarly, previous work by multiple groups has also demonstrated that both NOPR and MOR inhibit cAMP production and activate K^+ channels in a pertussis toxin (PTX)-sensitive manner (Matthes et al., 1996, Ma et al., 1997). In HEK293 cells, DOR have been shown to simultaneously inhibit the production of cAMP and induce p-ERK1/2 upon stimulation with morphine (Audet et al., 2008).

Interestingly, it has been recently reported that in rat corpus striatum acute stimulation of MOR and DOR suppresses the PKA pathway through both an inhibition of cAMP synthesis and cdk5-mediated inhibition of PKA (Ramos-Miguel and Garcia-Sevilla, 2012). Cdk5 is an enzyme which specifically inhibits the activated MEK1 and this therefore suggests controlled activation of ERK1/2 (Sharma et al., 2002). Exposure to chronic morphine in rats resulted in increased levels of MEK (both active and inactive forms) in the cortex, which could therefore lead to a dysregulation of ERK activation (Ramos-Miguel et al., 2011).

The endogenous opioid system includes several opioid peptides, which work on these receptors, including dynorphins, endorphins and enkephalins. These peptides are processed

within the cells from larger peptides found within the body. OLGs are capable of synthesizing the endogenous opioid peptides, proenkephalin and dynorphin (Knapp et al., 2001). Proenkephalin is subjected to differential posttranslational modifications dependent on age. Dynorphin has been shown to be involved in appetite, circadian rhythms (Przewlocki et al., 1983), stress responses (Land et al., 2008, Smith et al., 2012) and body temperature control (Xin et al., 1997).

Figure 6: Downstream mechanisms of the opioid and opioid-like receptors. While the downstream actions of the opioid and opioid-like receptors are still not fully understood, recent studies in multiple cell types have shown that these receptors may play a role in the regulation of calcium channels and the MAPK pathway through regulation of adenylate cyclase. [1](Audet et al., 2008), [2](Carter and Medzihradsky, 1993), [3](Kelamangalath et al., 2011), [4](Lee et al., 2011), [5](Ma et al., 1997), [6](Matthes et al., 1996), [7](Ramos-Miguel and Garcia-Sevilla, 2012), [8](Sharma et al., 2002), [9](Rhim and Miller, 1994)


NOPR
MOR
DOR
KOR
3
1, 2, 4,
5, 6, 7
9
1, 7
3
Adenylate cyclase
calcium channels
cAMP
neuron plasticicity
cdk5
7, 8
Raf-1
PKA
MEK
ERK
Cell growth,
proliferation,
differentiation

Nociceptin/orphanin FQ (**Figure 7**) is a widely expressed seventeen-amino acid peptide and the endogenous ligand for the nociceptin receptor (NOPR) (Meunier et al., 1995, Reinscheid et al., 1995). This peptide has structural similarities with opioids, such as dynorphin A, however, it lacks affinity for the classical opioid receptors and its action is unaffected by the opioid competitive antagonist naloxone (Meunier, 1997). In addition to its involvement in pain (Zeilhofer and Calo, 2003, Lambert, 2008), nociceptin has also been recently found to work in other physiological functions, such as the immune (Peluso et al., 1998, Fiset et al., 2003) and cardiovascular systems (Kapusta, 2000). Studies in rats and mice have shown that an intrahippocampal administration of the nociceptin peptide resulted in a dose-specific effect on spatial learning, with low doses improving and high doses impairing spatial learning (Sandin et al., 1997, Kuzmin et al., 2009). NOPR knockout mice were found to have improved learning and memory in a water maze test (Manabe et al., 1998).

The endogenous opioid system may also play a role in the onset and severity of myelin-related diseases, such as experimental autoimmune encephalomyelitis (EAE). One recent study has shown that Met(5)-enkephalin (opioid growth factor [OGF]), an endogenous opioid growth factor, was able to decrease signs of the disease, such as activated astrocytes and damaged nerves (Zagon et al., 2010). A follow up to this report found that mice exposed to OGF stopped the progression of EAE and was able to reverse the neurological signs associated with the disease such as wobbly gait and hind limb paralysis (Rahn et al., 2011).

Treatment and Abuse

Opioids, such as methadone and buprenorphine, are currently prescribed for pain management even in children and young adults (Anghelescu et al., 2011), at ages which correspond with secondary periods of rapid human brain myelination (Hunter et al., 1997, Paus et al., 1999). Therefore, it is plausible to hypothesize that these exogenous opioids may interfere with appropriate signaling from the endogenous opioid system and affect myelination.

Furthermore, between 2-6% of the patients prescribed with opioids on a long-term basis develop an addictive disorder (Christie, 2008). Epidemiological surveys show that opioids, including morphine, heroin and codeine, are among the most frequently abused drugs (Cicero et al., 2005, Compton and Volkow, 2006). A 2008 survey by the Substance Abuse and Mental Health Services Administration found that roughly 12 million people over 12 years of age had used a pain killer in a non-medical fashion (Butler et al., 2011). Among high school students, opioids such as Vicodin, are second only to marijuana in the rate of illicit drug use (Johnston, 2008). The magnitude of this problem is underscored by reports indicating that the trend of non-medical opioid use has increased in the USA by threefold since 1990 (Hall et al., 2008). The numbers of opioid drug abuse among youth are particularly alarming when considering the effects of exogenous opioids on nervous system development.

Treatment for opioid abuse includes substitution therapies with synthetic opioids such as buprenorphine and methadone. Buprenorphine, a semi-synthetic derivative of either thebaine or oripavine, is a known MOR partial agonist (Martin, 1979), a KOR antagonist (Leander, 1987) and, as our results show, also an NOPR agonist (Eschenroeder et al., 2012). As buprenorphine is

a partial agonist for MOR, ceiling effects are observed even with doses as high as 70 times the recommended dose being safely taken by non-dependent humans (Walsh et al., 1994). Additionally, buprenorphine has both a slow receptor association (30 minutes) and dissociation (50% bound after 1 hour) (Boas and Villiger, 1985).

Due to the positive history of the use of buprenorphine in opioid dependent patients, countries such as France have allowed the prescription of buprenorphine to opioid dependent patients by primary care physicians without additional training or a limiting number of patients prescribed with the drug (Auriacombe et al., 2004). In the United States, physicians with additional training can prescribe buprenorphine to opioid dependent patients in out-patient care, with limits on patient numbers (Fudala et al., 2003). However, buprenorphine does have potential for abuse and is additionally associated with withdrawal symptoms lasting for up to 10 days, though not as severe as those observed in the case of morphine (Mello et al., 1981).

Methadone is known to be a long-acting MOR agonist (Sim, 1973) and is the standard FDA approved treatment for pregnant opioid abusers. Methadone is typically used as part of a tapering treatment, where over time, its use increases completion of the program and assist with withdrawal symptoms (Amato et al., 2005).

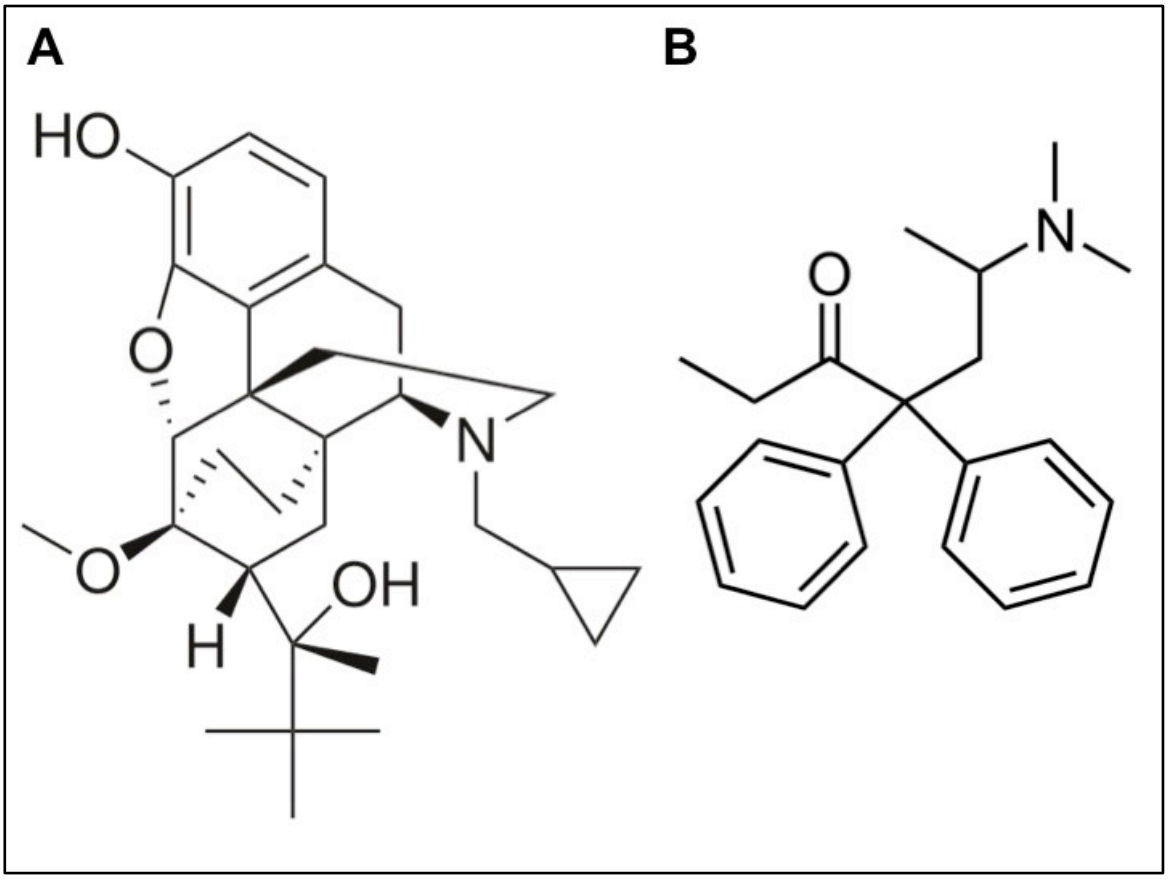
These two opioid agonists desensitize MOR and effectively reduce high affinity binding of agonists and this is in contrast to morphine, which does not work to desensitize the receptor. Therefore, buprenorphine and methadone may be more effective therapies in the treatment of opioid action due to this desensitization.

Opioid abuse and Pregnancy

The use of illicit substances during pregnancy in the United States is highly variable, with reports of use up to 27% (Bolnick and Rayburn, 2003). Additionally, 3% of women reported that their physicians prescribed them opioid painkillers for use during their pregnancy. Synthetic opioids, such as buprenorphine and methadone (**Figure 7**), are currently used as therapy for pregnant opioid addicts with the goal of reducing the amount of "street drug" use and ensuring a safer pregnancy for the mother and child. Clinical trials with buprenorphine treatment have shown a reduction in neonatal abstinence syndrome (Jaffe and O'Keeffe, 2003, Amass et al., 2004) and methadone administration was shown to decrease heroin exposure for the fetus and to eliminate risks of fetal withdrawal (McCarthy, 2012). However, methadone treatments appeared to be less successful in decreasing addiction with reports of up to 80% of the prescribed pregnant patients continuing to use illicit drugs in addition to methadone.

Studies of infants exposed to exogenous opioids *in utero* reported reduced head circumference, decreased attention, altered fine motor coordination and a greater risk for Sudden Infant Death Syndrome (Cohen et al., 1982, Johnson and Rosen, 1982, Rosen and Johnson, 1982, Kandall et al., 1993, Jansson et al., 2011a), suggesting that the developing nervous system may be affected. Additionally, infants exposed to methadone have been found to have a reduced baseline heart rate and decreased motor activity (Jansson et al., 2011b). However, cognitive and behavioral effects of opioid exposure in these children are difficult to dissociate from those that may on the other hand result from a negative environment.

Figure 7: Synthetic opioids used in the treatment of opioid abuse patients. Chemical structure of **(A)** buprenorphine and **(B)** methadone. These synthetic opioids are currently used in the treatment of opioid addiction and management of pain.


A
B
HO
O
N
O
OH
H
O
N

Importantly, the effects of buprenorphine or methadone exposure *in utero* on brain development have not been thoroughly studied. However, this is a problem of great clinical significance, as most brain cells are known to express opioid receptors. As described before, OLGs also express multiple opioid receptors (Knapp et al., 1998) and thus, binding of methadone, buprenorphine or other exogenous opioids, could interfere with the endogenous opioid system perhaps altering OLG maturation and myelination, and possibly affecting brain development.

Furthermore, as indicated above, it is a currently accepted medical practice to prescribe women with opioids to relieve their pain during pregnancy and as such, it is critical to fully understand how these exogenous opioids could affect the developing brain of the fetus. The mammalian placenta serves to connect the circulatory systems of the mother and child, allowing for the transfer of gases and nutrients. However, the traditional assumption that exogenous drugs would not pass from the mother to the child through the placenta has now been shown to be incorrect (Syme et al., 2004). Many drugs can rapidly cross the placenta by both active and passive transfer. After birth, children could continue to be exposed to these drugs through the breast milk or may likely need to be administered methadone or buprenorphine to ameliorate their own opioid withdrawal symptoms.

Importantly, previous results from this laboratory showed that perinatal exposure to buprenorphine can affect myelination in the developing rat brain (Sanchez et al., 2008). In these studies, rat pups were first exposed to buprenorphine through the placenta and then through lactation. Analysis of these rat brains showed that therapeutic doses of buprenorphine

accelerated and increased the expression of MBPs, while higher doses resulted in a delay in protein expression **(Figure 8)**. Interestingly, regardless of the dose of buprenorphine, analysis of the corpus callosum demonstrated increased axonal caliber accompanied by a thinner myelin sheath, suggesting an opioid effect on the interactions between the axon and OLG.

Through the results of buprenorphine exposure in isolated, cultured OLGs, this laboratory next showed that the *in vivo* effects on myelination could result from direct drug action on the developing cells through activation of opioid or opioid-like receptors **(Figure 9)** (Eschenroeder et al., 2012). Similar to the previously observed *in vivo* results, direct exposure of the cells to buprenorphine in culture also resulted in a bell-shaped dose-specific response in the protein levels of MBP. Low buprenorphine doses elicited a significant increase in the expression of MBP, with a maximal stimulation occurring between 0.25 μM and 0.5 μM. However, the capacity of buprenorphine to up-regulate MBP expression is lost at higher drug concentrations (**Figure 9**).

Figure 8: Perinatal exposure to buprenorphine alters the timing and levels of MBP expression. Rat pups were perinatally exposed to buprenorphine (0.3 or 1mg/kg/day) via mini pump. Water was used for the control pups. Levels of 14 kDa, 17 kDa, 18.5 kDa and 21.5 kDa isoforms of MBP in total brain homogenates were determined by western blot analysis at 12 (**A**), 19 (**B**) or 26 days (**C**) post-natal. The results are expressed as a percentage of the control values and are the mean ± SEM from at least 12 brains from 3 different litters. *p<0.05, **p<0.005, ***p<0.0005. Taken from Sanchez et al., 2008.

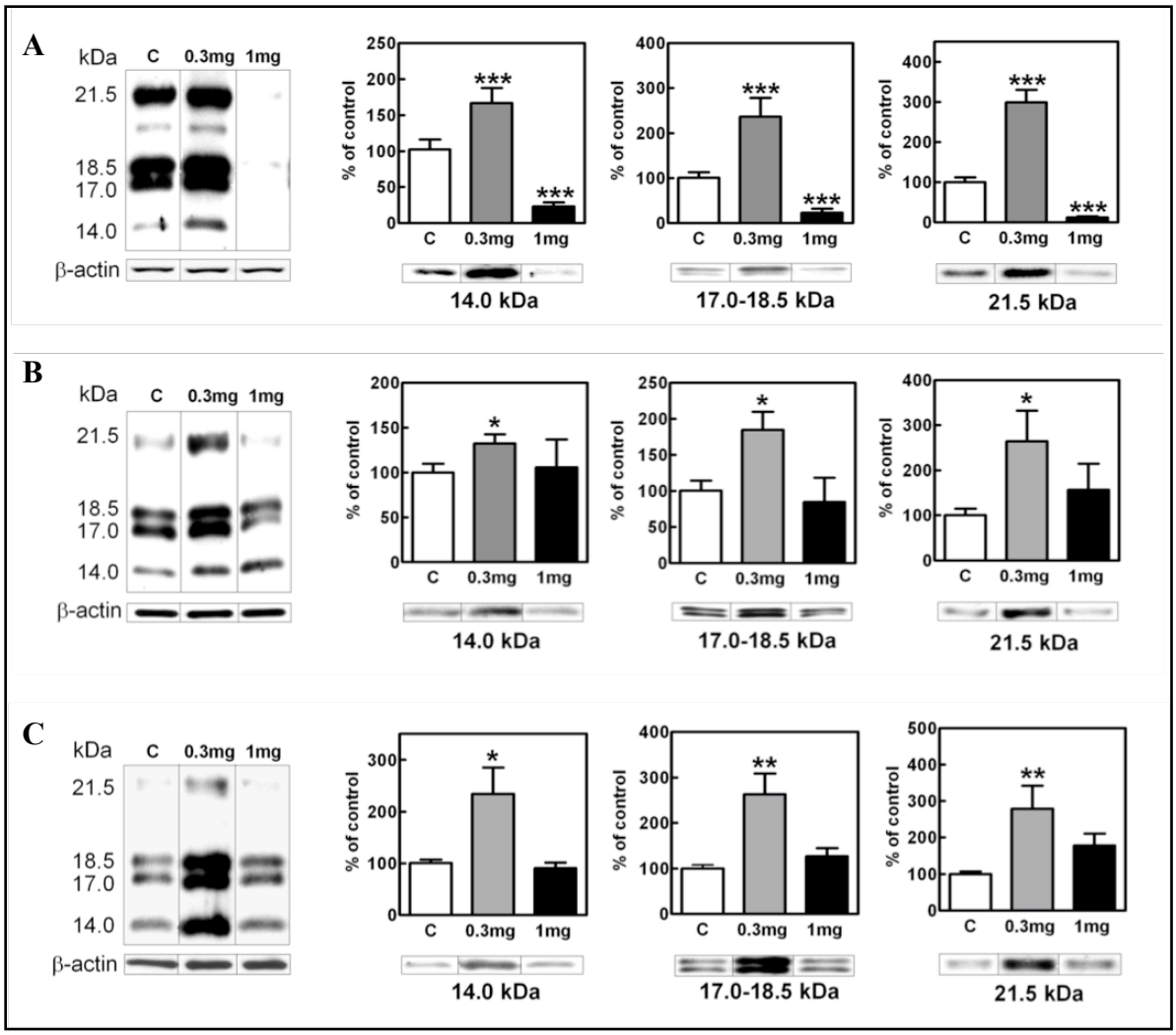
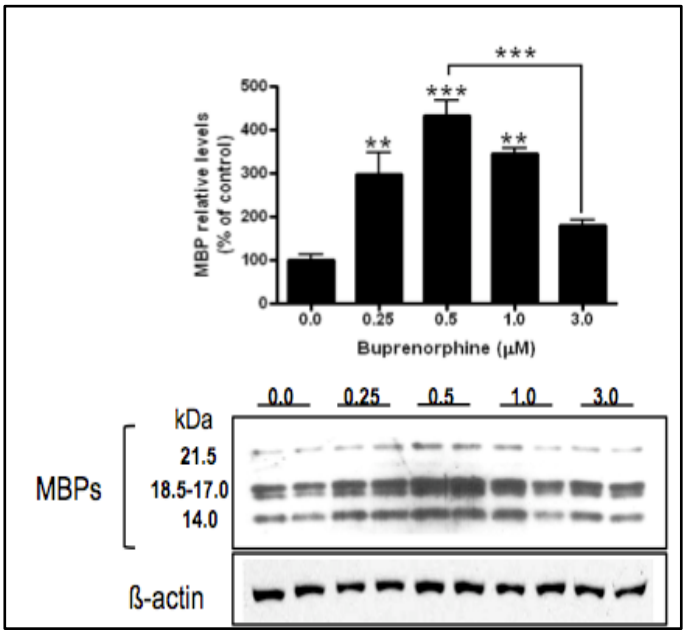

A
kDa
C
0.3mg
1mg
21.5
18.5
17.0
14.0
β-actin
% of control
***
14.0 kDa
17.0-18.5 kDa
21.5 kDa
B
*
C
C
*
**

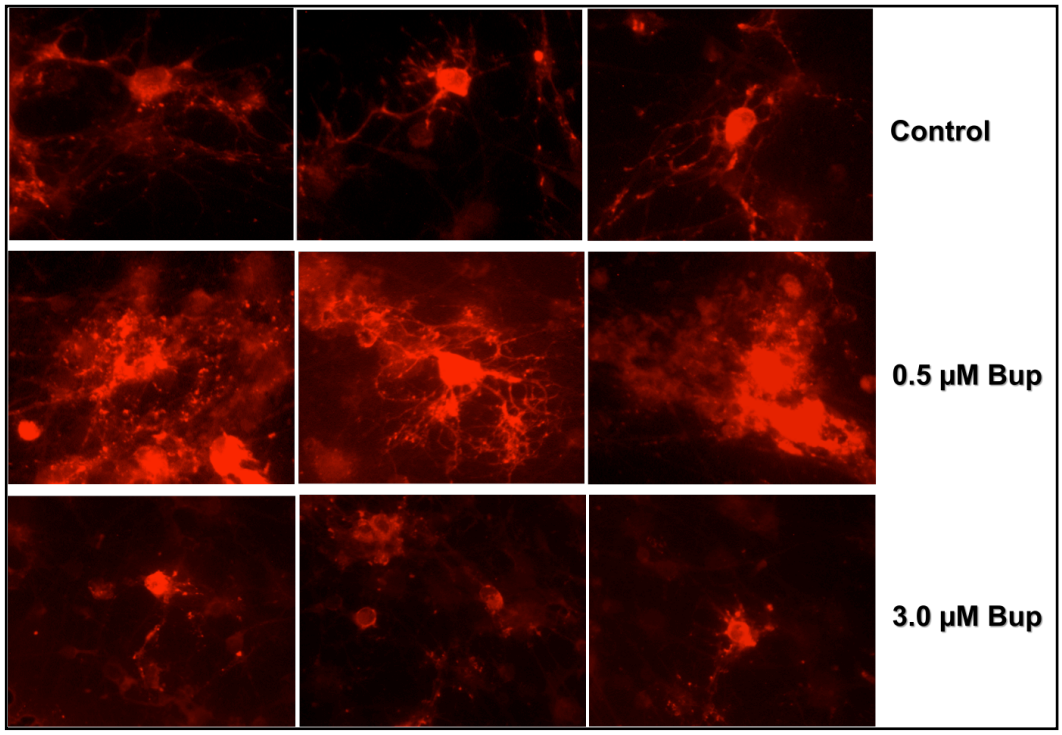
Figure 9: Buprenorphine has direct effects in OLGs, causing changes in MBP expression in a dose-dependent manner. OLGs were isolated from 9-day-old rats and cultured for 4 days in chemically defined medium (CDM). Cells were incubated with 0.25, 0.5, 1.0 or 3.0 μM buprenorphine and compared to controls in CDM alone. Levels of MBP were determined by western blot analysis. Levels of ß-actin were used as loading controls. MBP values of the four splicing variants were combined. **p<0.005, ***p<0.0001. Taken from Eschenroeder et al., 2012.


***
***
**
**
500
400
300
200
100
0
MBP relative levels
(% of control)
0.0
0.25
0.5
1.0
3.0
Buprenorphine (μM)
0.0
0.25
0.5
1.0
3.0
kDa
21.5
18.5-17.0
14.0
MBPs
ß-actin

Therefore, while exogenous opioids could potentially work on multiple cell types with the appropriate opioid receptors, these results show that buprenorphine can indeed exert direct effects on the OLGs.

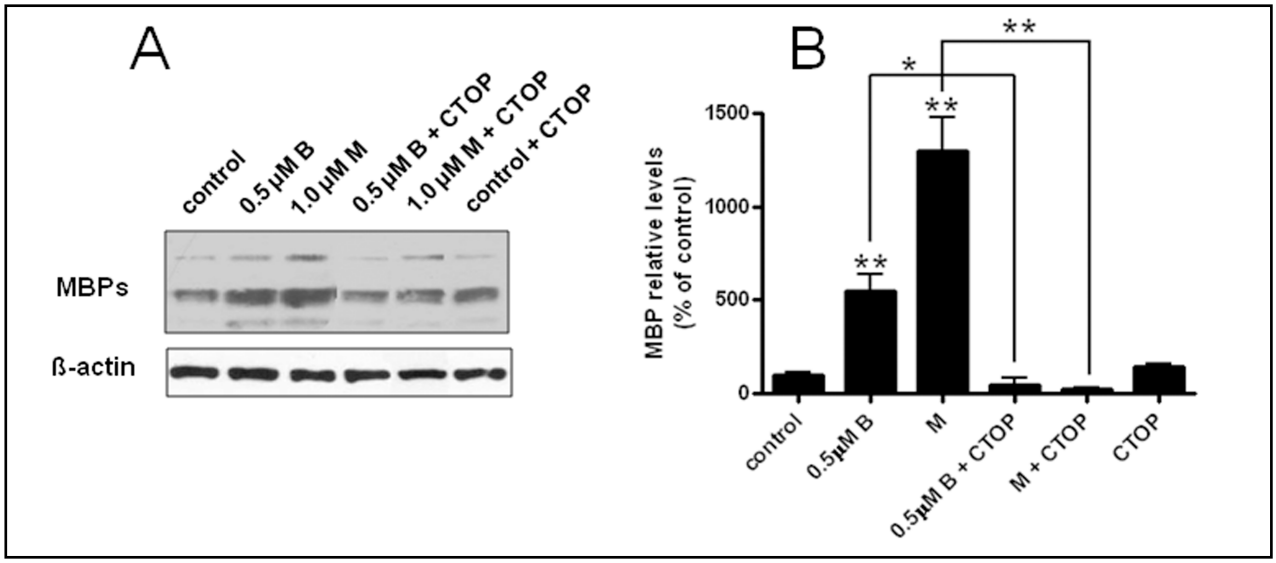
Furthermore, by using immunocytochemistry with anti-MBP antibody, we found that exposure to 0.5 μM buprenorphine not only resulted in significantly increased MBP expression but this effect was accompanied by a more complex phenotype, with greater process extension and membrane outgrowth indicative of highly mature OLGs **(Figure 10)**. In contrast, lower MBP levels, in control cells and OLGs exposed to the higher buprenorphine dose, coincided with cells exhibiting shorter processes and overall simpler morphology.

Figure 10: OLG process extension and membrane growth are affected in a dose-dependent manner by buprenorphine. OLGs were isolated from 9-day-old rats and cultured for 4 days in chemically defined medium alone or with 0.5 μM or 3.0 μM buprenorphine. Cells were stained for MBP and visualized by fluorescent microscopy. Representative fields are shown for each treatment. Taken from Eschenroeder et al., 2012.


Control
0.5 μM Bup
3.0 μM Bup

In addition, our previous results also demonstrated that the stimulatory effects induced by 0.5 μM buprenorphine are mediated by MOR. As shown in **Figure 11**, MBP expression in the OLGs is also stimulated by the MOR-specific ligand methadone, and both methadone and 0.5 μM buprenorphine positive effects are eliminated by co-incubation with the MOR specific antagonist CTOP.

Figure 11: The stimulatory effect of buprenorphine is mimicked by methadone and both drug effects are abolished by the MOR antagonist, CTOP. Pre-OLGs from 9-day-old rat brain were cultured for 4 days in the presence or absence of 1 μM methadone (1 μM M) or 0.5 μM buprenorphine (0.5 μM B) with or without 1 μM CTOP. **(A)** MBP levels were determined by western blot analysis. **(B)** Results in the bar graph correspond to the combined scanning of the four major MBP isoforms from at least three experiments and are expressed as percentage of controls (0 μM buprenorphine) ± SEM. $*P < 0.02$ and $**P < 0.0001$. Taken from Eschenroeder et al., 2012.


A
control
0.5 μM B
1.0 μM M
0.5 μM B + CTOP
1.0 μM M + CTOP
control + CTOP
MBPs
ß-actin
B
MBP relative levels
(% of control)
1500
1000
500
0
*
**
**
**
control
0.5μM B
M
0.5μM B + CTOP
M + CTOP
CTOP

The lack of stimulatory effects at high buprenorphine doses was not due to cell death by toxicity (Eschenroeder et al., 2012). On the other hand, it is possible to speculate on a mechanism due to receptor desensitization induced by high agonist concentration or perhaps down-regulation of MOR, as observed with prolonged exposure to morphine (Zhu et al., 2012). Additionally, long-term activation of MOR by the endogenous peptide enkephalin results in receptor degradation in the lysosome (Hislop et al., 2011).

However, as described in the following sections, the studies in this thesis work showed that the bell-shaped effects of buprenorphine on the OLGs could be explained by a balance between opposing effects mediated by MOR and NOPR. The following work will describe how this laboratory's previous results, demonstrating a decrease in MBP expression with high levels of buprenorphine, are due to a concurrent activation of the NOPR. This opioid receptor-like receptor has been previously shown to compromise the anti-nociceptive effect of buprenorphine when it is activated along with MOR (Lutfy et al., 2003, Khroyan et al., 2009) and blocking NOPR facilitated the rewarding effect of conditioned place preference due to morphine (Rutten et al., 2011). Interestingly, MOR and NOPR have been shown to have opposing effects on immune function, with MOR activation favoring a pro-inflammatory response and NOPR activation playing a role in the down-regulation of immune function (Finley et al., 2008).

The following experiments in this thesis will demonstrate that OLGs also express NOPR and that activation of MOR and NOPR do result in opposing effects on OLG differentiation and therefore, myelin protein expression. We have found that activation of MOR by low doses of buprenorphine or methadone result in an increase in the percentage of differentiated,

myelinating OLGs and in an increase in the expression of MBP. However, upon concurrent activation of NOPR, by higher doses of buprenorphine or by specific NOPR ligands, such as the endogenous ligand for NOPR, nociceptin, this increase in both OLG differentiation and the expression of myelin proteins is abolished. Interestingly, further study of this receptor system may provide new clues about the role of the endogenous opioid system in OLG differentiation and how exposure to exogenous opioids during critical development periods may affect the OLGs and myelination.

MATERIALS AND METHODS

Materials

Percoll, bovine pancreas DNAse, papain for cell isolation and cell culture medium components were from Sigma–Aldrich (St. Louis, MO). Dulbecco's modified Eagle's medium/Ham's F-12 (DMEM/F-12) (1:1) medium with high glucose and L-glutamine was obtained from Invitrogen (Grand Island, NY). Reduced-growth factor Matrigel was from Becton Dickinson (Franklin Lakes, NJ). Buprenorphine, methadone, and the MOR antagonist CTOP (D-Phe-Cys-Tyr-D-Trp-Orn-Thr-PenThr-NH2 [Disulfide Bridge: 2–7]) were purchased from Sigma–Aldrich (St. Louis, MO). The NOPR inhibitor J113397 (1-[(3R*,4R*)-1-(Cyclooctylmethyl)-3-(hydroxymethyl)-4-piperidinyl]-3-ethyl-1,3-dihydro-2H-benzimidazol-2-one) and nociceptin (Orphanin FQ) were from Tocris Bioscience (Ellisville, MO). All gel electrophoresis reagents and supplies were purchased from Bio-Rad Laboratories (Hercules, CA). Anti-ß-actin and anti-MBP (82–87 region) antibodies were from Sigma– Aldrich and Millipore Corporation (Temecula, CA), respectively. The MOR and NOPR antibodies were from Neuromics (Edina, MN). The mouse O4 monoclonal antibody was kindly provided by Dr. Rashmi Bansal (University of Connecticut, Farmington, CT) and Dr. Babette Fuss (Virginia Commonwealth University, Richmond, VA). The super Signal West Dura chemiluminescence reagent and protease inhibitor cocktail were from Pierce-Thermo (Rockford, IL). All appropriate secondary antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Model 2ML4 Alzet osmotic minipumps were from Durect (Cupertino, CA).

Oligodendrocyte Isolation

Pre-OLGs were isolated from the 9-day-old Sprague-Dawley rat brain using Percoll gradient centrifugation (Berti Mattera et al., 1984) and differential adhesion as previously described (Saini et al., 2005). Animal use and OLG isolation were conducted in accordance with the guidelines from the National Institutes of Health and approved by the Virginia Commonwealth University Animal Care and Use Committee.

After sacrifice by decapitation and brain isolation, the meninges and blood vessels were quickly removed by gently rolling the tissue on sterile filter paper. The tissue was finely minced and incubated with papain (1 unit/mL) and DNAse I (0.01 mg/ml) in HEPES/HBSS solution, for 25 min at 37°C. The tissue was then centrifuged and the resulting pellet was washed with DNAse I solution and HEPES/HBSS solution. The cells were filtered through a nylon mesh (75 μm pore size) and were then centrifuged in an isotonic Percoll gradient for 15 min at 30,000 x g. The band containing the OLGs was collected, diluted 3-4 times with HEPES/HBSS and centrifuged for 10 min at 300 x g. The resulting pellet was suspended in DMEM/F-12 and transferred to a tissue-treated Petri dish and allowed to incubate undisturbed for differential adhesion of microglia and residual astrocytes. At the end of the incubation period, the dish was swirled lightly to recover in the medium the suspended OLGs. After centrifugation, the pellet was then re-suspended in the appropriate volume of chemically defined medium, as determined by a cell number count with a hemacytometer.

Cell Culture

The isolated OLGs were plated on Matrigel-coated 24-48 microwell Falcon plates (for western blot analysis) or 8-well slide Permanox chambers (for immunocytochemistry). Matrigel resembles the natural tissue extracellular environment, containing extracellular matrix proteins such as laminin and collagen (Kleinman et al., 1986). The cells were then cultured for different times in chemically defined medium (CDM) (DMEM/F12 with 1 mg/mL fatty acid-free bovine serum albumin, 100 µg/mL transferrin, 5 µg/ml insulin, 30 nM sodium selenite, 0.11 mg/ml sodium pyruvate, 10 nM biotin, 20 nM progesterone, 100 µM putrescine and 30 nM triiodothyronine), in the presence or absence of different concentrations of buprenorphine, methadone, or receptor inhibitors as later indicated in the text.

Western Blot Analysis

For western blot analysis, cell cultures were lysed in 50-100 µl of 60mM Tris-HCl buffer (pH 6.8) containing 10% glycerol, 2% sodium dodecyl sulfate (SDS) and 5% 2-mercaptoethanol. Lysates were then subjected to SDS-polyacrylamide gel electrophoresis (SDS-PAGE) in 12 or 15% acrylamide and the proteins electro-transferred to nitrocellulose, as previously described (Saini et al., 2005). The nitrocellulose membranes were then used for immunoblot analysis. Non-specific binding was blocked by incubation in 10 mM Na_2HPO_4, 2.7 mM KCl and 137 mM NaCl, pH 7.4 (PBS), with 3% nonfat dry milk and 0.05% Tween-20 (blocking solution) for 1 hour at room temperature. The membranes were then incubated overnight in blocking solution containing the appropriate concentration of primary antibody. These concentrations were 1:100 for anti-MBP, 1:500 for anti-MOR, 1:500 for anti-NOPR and

1:2,000 for anti-ß-actin. Blots were then rinsed in PBS three times before a second blocking period of 30 minutes. The blots were then incubated with the appropriate horseradish peroxidase-conjugated secondary antibody in blocking solution for 3 hours. The blots were then washed twice with PBS and 0.05% Tween-20 and then at least four times with PBS alone. The immunoreactive bands were detected by chemiluminescence with Super Signal West Dura reagent and the relative amount of protein in each band was determined by scanning densitometric analysis of the films using the NIH ImageJ program. The resulting protein levels were then divided by the β-actin levels measured for each sample to correct for any possible differences in loading amount.

Immunocytochemistry

Cells were fixed in 4% paraformaldehyde in PBS, and immunocytochemistry was carried out as previously reported (Sato-Bigbee et al., 1999). Nonspecific antibody binding was blocked by incubation of the cultures for 1 h in PBS containing 5% nonfat dry milk, 0.05% Tween-20, and 0.5% normal goat serum (blocking solution). The cells were then incubated overnight with a mixture of O4 (dil.1:3) and anti-MBP (dil. 1:20) antibodies in blocking solution. After several washes in PBS, the cells were incubated for 30 min in blocking solution and for 2 h with Alexa 488-conjugated anti-mouse IgM (dil. 1:250) and Texas Red-conjugated anti-rat IgG (dil. 1:150) for O4-MBP double immunocytochemistry.

For detection of MOR and NOPR, cells were incubated overnight in a mixture of O4 and anti-MOR (dil. 1:100) or anti-NOPR (dil. 1:100) antibodies in PBS containing 0.1% Triton X-100 and 1% normal goat serum. After extensive washing and a second 30 min blocking step,

cells were incubated for 2 h with a mixture of Alexa 488-conjugated anti-mouse IgM (dil. 1:250) for O4 labeling and Texas Red-conjugated anti-rabbit IgG (dil. 1:150) for MOR and NOPR visualization. The cultures were then analyzed using a Nikon Eclipse 800 M fluorescence microscope with ad hoc digital camera system.

Methadone Treatment

Rat pups were exposed to methadone pre- and post-natally. On day 7 of gestation, dams were implanted subcutaneously, while under isoflurane anesthesia, with 28-day osmotic minipumps to deliver sterile water (controls) or methadone HCl (9 mg/kg/day) as previously described (Robinson and Wallace, 2001). Therefore, exposure to the drug occurs prior to CNS development and continues, via maternal milk, until time of sacrifice or weaning. Pups were sacrificed for analysis at the postnatal days 11 and 19.

Analysis of *In Vivo* Myelination

To determine the extent of *in vivo* myelination, 19-day-old pups were anesthetized via intraperitoneal injection with 2.5% Avertin and perfused transcardially with 100mM phosphate buffer, pH 7.3, containing 4% paraformaldehyde and 3.5% glutaraldehyde as previously described (Dupree et al., 2004). The brains were removed and maintained in the above solution for 3-5 days. The area containing the corpus callosum was dissected, postfixed with 1% osmium tetroxide in 100mM sodium cacodylate buffer, pH 7.3 and plastic embedded. One micron sections were stained with toluidine blue and were photographed at 1,000X magnification.

For analysis of *in vivo* MBP expression, 11- and 19-day-old pups were sacrificed by decapitation, the brains rapidly removed, transferred to ice and homogenized in 10 mL of ice-cold PBS supplemented with a protease inhibitor cocktail, using a Potter-Elvehjem tissue grinder. After protein determination, adequate aliquots were subjected to SDS-PAGE and western blot analysis with an anti-MBP antibody as indicated above, using ß-actin levels as loading control.

Statistical Analysis

For each of the following studies, the experiments were repeated in duplicate at least 3 times and the results were analyzed by one-way ANOVA, using the GraphPad Prism program. Results were considered statistically significant when $p<0.05$.

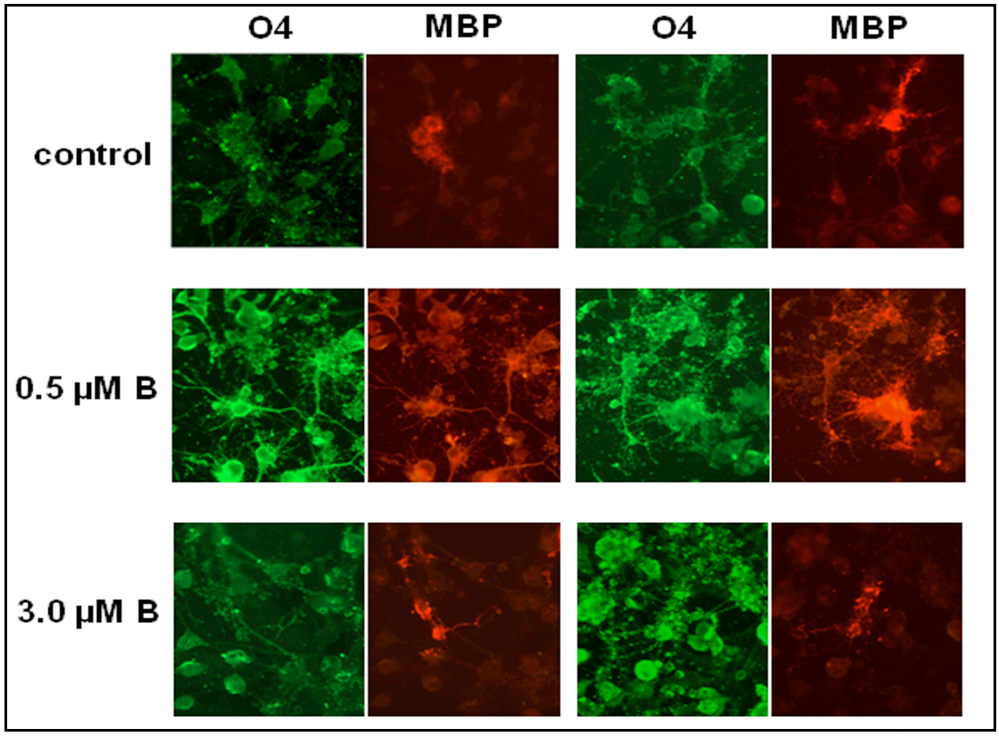
RESULTS

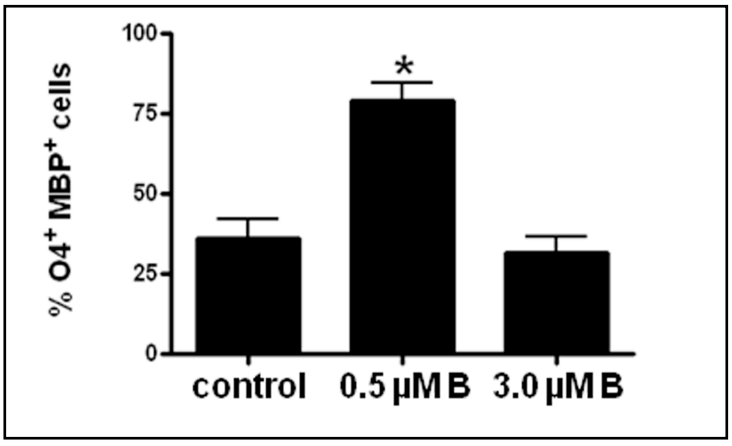
Buprenorphine alters the number of cells that reach the mature, MBP-expressing developmental stage in a dose-specific manner.

As discussed before, our previous observations showed that perinatal exposure to buprenorphine results in bell-shaped effects on MBP brain expression (Sanchez et al. 2008) and these observations are mirrored by direct effects of buprenorphine on the OLGs (Eschenroeder, 2012). Thus, we next sought to determine whether these results could be due to dose-specific effects on the percentage of cells differentiating into mature OLGs capable of myelination or instead be the reflection of OLGs expressing different levels amount of MBP protein per cell. In order to answer to this question and better understand the effect of the different buprenorphine doses on the OLGs, we analyzed the percentage of immature cells that were able to transition into mature MBP positive OLGs. For this, cells were analyzed by double immunocytochemistry with O4 and MBP antibodies (**Figure 12**). As previously depicted in **Figure 5** of the Introduction section, cells labeled by the O4 monoclonal antibody represent pre-OLGs, while cells labeled by both O4 and MBP are representative of more differentiated OLGs, capable of myelinating an axon *in vivo*.

Analysis of the cell cultures by double immunocytochemistry with O4 and MBP antibodies (**Figure 12**) further supported the idea that the previously described observations indeed reflect differences in the extent of cell differentiation. Although both immature pre-OLGs and mature cells are known to react with the O4 monoclonal antibody, only differentiated OLGs are expected to be labeled by both the O4 and the MBP antibodies. In the presence of 0.5 μM buprenorphine, the great majority of the cells are both highly O4 and MBP positive. In contrast, controls incubated in the absence of buprenorphine or cells treated with the higher 3.0 μM buprenorphine dose exhibited a significant number of cells that were solely O4 positive, but significantly less that were highly labeled by both the O4 and MBP antibodies as compared to the cells exposed to 0.5 μM buprenorphine. Quantification of these results (**Figure 12**, bar graph) indicated that in cultures exposed to 0.5 μM buprenorphine roughly 80% of the cells are labeled by both the O4 and MBP antibodies, whereas this number is only about 35% in the case of controls and cells treated with 3.0 μM buprenorphine. Taken together, the above observations are consistent with the original hypothesis that buprenorphine exerts direct effects on OLG maturation. In addition, the bell-shaped dose-dependent nature of the drug effects indicates the existence of a complex mechanism. Therefore, we next investigated the basis for these dose-specific effects of buprenorphine on the OLGs.

Figure 12: Buprenorphine affects OLG differentiation in a dose-dependent manner. Pre-OLGs isolated from 9-day-old rat brain were cultured for 4 days in CDM alone (control) or supplemented with 0.5 μM buprenorphine (0.5 μM B) or 3.0 μM buprenorphine (3.0 μM B). After 4 days of treatment, cells were analyzed by double immunocytochemistry with O4 (green) and anti-MBP (red) antibodies. Notice that cells that are both O4 and highly MBP positive are particularly increased in the cultures exposed to 0.5 μM buprenorphine. Scale bar: 20 nm. The bar graph indicates the percentage of O4 positive cells that are also MBP positive under each experimental condition. The results represent the average +/- SEM from 5 different fields and at least 100 cells per condition. *$p<0.001$.


O4
MBP
O4
MBP
control
0.5 μM B
3.0 μM B


% O4+ MBP+ cells
100
75
50
25
0
*
control
0.5 μM B
3.0 μM B

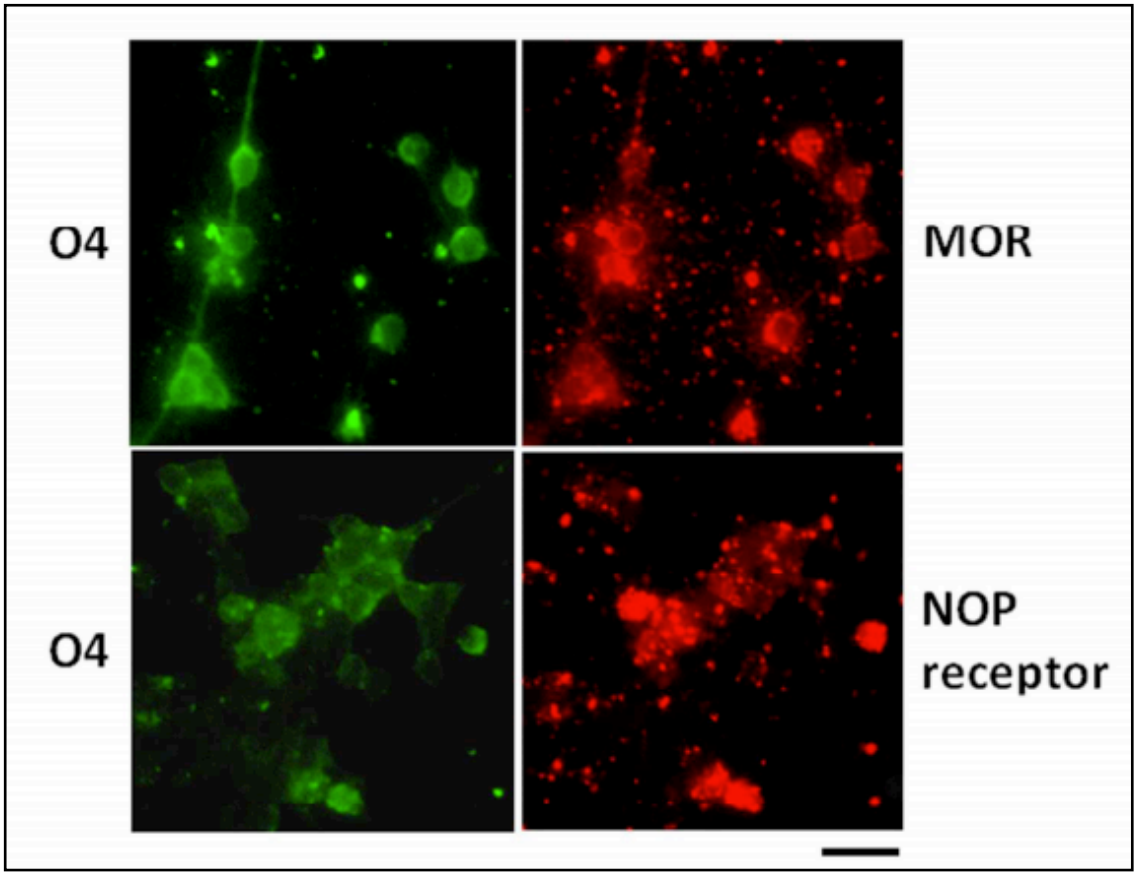
Pre-OLGs express both MOR and NOPR

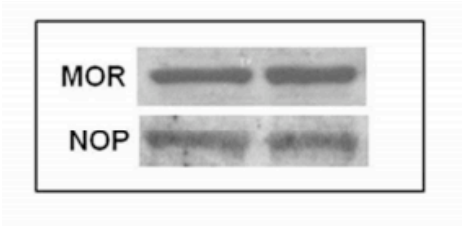
Our previous results using methadone and the MOR inhibitor CTOP pointed to 0.5 μM buprenorphine working through activation of MOR (**Figure 11**). However these observations did not explain the differential effect of the higher 3 μM dose of buprenorphine. Interestingly, similar to the dose-specific effects on MBP expression and OLG development described above, others have shown that buprenorphine antinociceptive effects also exhibit a bell-shaped dose-response. Low buprenorphine concentrations exert an analgesic effect but this antinociceptive action is significantly decreased by higher levels of the drug (Dum and Herz, 1981, Lizasoain et al., 1991). Later investigations also found that antinociception induced by buprenorphine is mediated by MOR but higher doses counteract this effect by concomitant activation of the NOPR (Lutfy et al., 2003), a molecule that has about 75% homology with the three different opioid receptors (Mogil and Pasternak, 2001).

However, the expression of the NOPR in OLGs had not been previously reported. To address this problem, we used fluorescent double-immunocytochemistry with O4 together with NOPR or MOR antibodies to determine the presence of these receptors in the pre-OLGs.

We found that the pre-OLGs isolated from 9-day-old rat brain not only expressed MOR (as previously reported by (Knapp et al., 1998)), but also NOPR (**Figure 13**), with both receptors being detected by immunocytochemistry and in addition by western blot analysis.

Figure 13: Pre-OLGs express both the MOR and the NOP receptor. Cells isolated from 9-day-old rat brain were allowed to fully attach on the culture plates by overnight incubation and stained by double immunocytochemistry with O4 (green) together with anti-MOR or anti-NOP receptor antibodies (red). Scale bar: 20 μm. The western blot shows MOR and NOP receptor expression in two different samples of developing oligodendrocytes directly isolated from 9-day-old rat brains.


O4
MOR
O4
NOP
receptor


MOR
NOP

Nociceptin receptor inhibition restores MBP up-regulation in the presence of high buprenorphine doses

In view of the above results, we decided to investigate if NOPR could be responsible for the effects observed when OLGs are cultured in medium containing high doses of buprenorphine. For this, we next treated the cells with 3 μM buprenorphine together with the NOPR specific inhibitor J-113397 (Kawamoto et al., 1999). If the lack of increase in MBP expression and OLG differentiation in the presence of high doses of buprenorphine was indeed due to NOPR activation, co-incubation with an NOPR inhibitor should restore the stimulation resulting from MOR activation alone.

The results in **Figure 14** indeed suggest that an antagonistic functional relationship between MOR and NOPR also exists in the OLGs and underlies the dose-specific effects of buprenorphine on these cells. Analysis of MBP expression showed that the bell-shaped dose-dependent effect of buprenorphine is abolished by inhibition of NOPR. As shown in **Figure 14**, cultures exposed to 3 μM buprenorphine in the presence of J-113397 exhibited significantly higher MBP levels than both controls and cells exposed to 3 μm buprenorphine alone. This suggests that inhibition of NOPR allows for buprenorphine to act solely on MOR, resulting in increased MBP expression as previously reported **(Figure 11)**. This is in contrast with the MBP expression in cells treated with 3 μM buprenorphine and the MOR inhibitor CTOP, which exhibited MBP levels that were even lower than in controls (**Figure 15**).

Figure 14: NOPR down-regulates the expression of MBP in response to high doses of buprenorphine. Cells isolated from 9-day-old rat brain were cultured for 4 days in CDM in the presence or absence of 3 μM buprenorphine (3μM B) and increasing concentrations of the NOP receptor inhibitor J-113397 (J). Results in the bar graph are expressed as percentage of controls (0μM buprenorphine) ± SEM from at least 5 experiments. *P < 0.02 and **P < 0.005. Results are expressed as percentage of controls (0μM buprenorphine) ± SEM from four experiments and correspond to the combined scanning of the four major MBP isoforms.

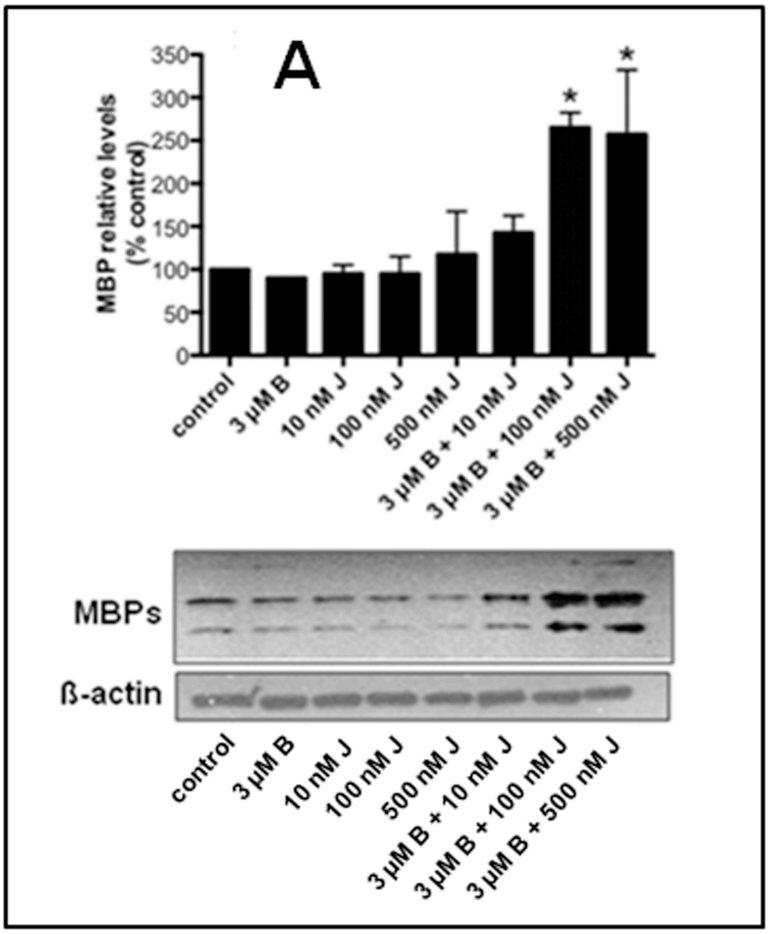
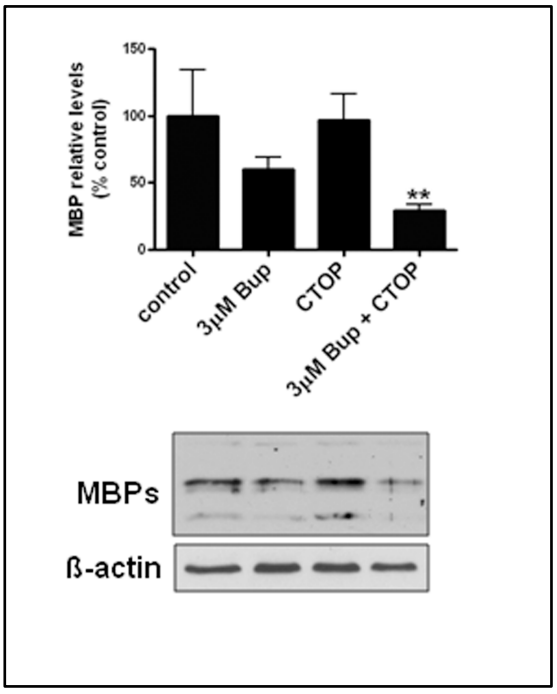

A
MBP relative levels (% control)
350
300
250
200
150
100
50
0
*
*
control
3 µM B
10 nM J
100 nM J
500 nM J
3 µM B + 10 nM J
3 µM B + 100 nM J
3 µM B + 500 nM J
MBPs
ß-actin
control
3 µM B
10 nM J
100 nM J
500 nM J
3 µM B + 10 nM J
3 µM B + 100 nM J
3 µM B + 500 nM J

Figure 15: Activation of NOPR with concurrent MOR inhibition result in further reduced expression of MBP. Cells isolated from 9-day-old rat brain were cultured for 4 days in CDM in the presence or absence of 3 μM buprenorphine with or without the MOR inhibitor CTOP. Results in the bar graphs are expressed as percentage of controls (0μM buprenorphine) ± SEM from at least 5 MBP isoforms. *P < 0.02 and **P < 0.005. Results are expressed as percentage of controls (0μM buprenorphine) ± SEM from four experiments and correspond to the combined scanning of the four major MBP isoforms.


MBP relative levels
(% control)
150
100
50
0
**
control
3μM Bup
CTOP
3μM Bup + CTOP
MBPs
ß-actin

The endogenous peptide for NOPR, nociceptin, opposes the stimulatory effects of both low buprenorphine doses and methadone

The results described above supported the idea that NOPR opposes the stimulatory effects on OLG maturation exerted by MOR. To further confirm the role of NOPR in the OLG response to high levels of buprenorphine and to better understand the role of endogenous ligands in this system, OLGs were directly exposed to the endogenous peptide for NOPR, nociceptin. We hypothesize that a combination of the low, stimulatory dose of 0.5 μM buprenorphine with nociceptin, would mimic the results obtained with the 3 μM dose of buprenorphine if this higher dose is indeed exerting an inhibitory effect by additionally binding to NOPR.

For this, cells were cultured for 4 days in either control medium or medium supplemented with (1) nociceptin, (2) 0.5μM buprenorphine, or (3) nociceptin and 0.5 μM buprenorphine. The results indicated that MBP expression in OLGs exposed to nociceptin is comparable to that of control cells, and as before, the expression of these proteins is greatly increased in cells stimulated with the low dose of buprenorphine alone (**Figure 16**). However, treatment of these cells with nociceptin abolished this stimulatory effect of 0.5 μM buprenorphine, which as described above is mediated by MOR. These results further suggested that MOR and NOPR have opposing effects on MBP expression and an inhibitory action mediated by NOPR is responsible for lack of effects observed at high doses of buprenorphine.

This relationship between MOR and NOPR was further explored with methadone, which is known to specifically exert its effects through MOR. OLGs were cultured with this drug both with and without nociceptin (**Figure 17**). The results showed that the "antagonistic" role of MOR and NOPR on MBP expression is not only observed for buprenorphine but also for the MOR-specific ligand methadone. As shown in **Figure 17**, the stimulatory effect of methadone on MBP expression is counteracted by co-incubation of the cells with nociceptin.

However, as observed before in **Figure 16**, MBP levels are not altered by nociceptin alone. This later observation is particularly important because it indicates that, rather than working as part of an independent inhibitory pathway, NOPR is functionally related to MOR activity. Altogether, these findings support the existence of a MOR/NOPR regulatory system in which signaling through MOR and NOPR exert opposite effects on OLG development. Nociceptin is expressed throughout the CNS and has been shown to be produced by both neurons (Mika et al., 2011) and astrocytes (Buzas et al., 1998). Therefore, it is tempting to theorize that this endogenous peptide may play a role in the regulation of the timing of myelination.

Figure 16: The endogenous peptide for NOPR abrogates stimulation of MBP expression by the low dose of buprenorphine. Cells were cultured in the presence or absence of 0.5 μM buprenorphine (0.5 μM B) with or without 1μM nociceptin. Results are expressed as percentage of controls (0 μM buprenorphine) ± SEM from four experiments and correspond to the combined scanning of the four major MBP isoforms. *P <0.05. MBP levels were determined by western blotting using b-actin levels as loading controls.

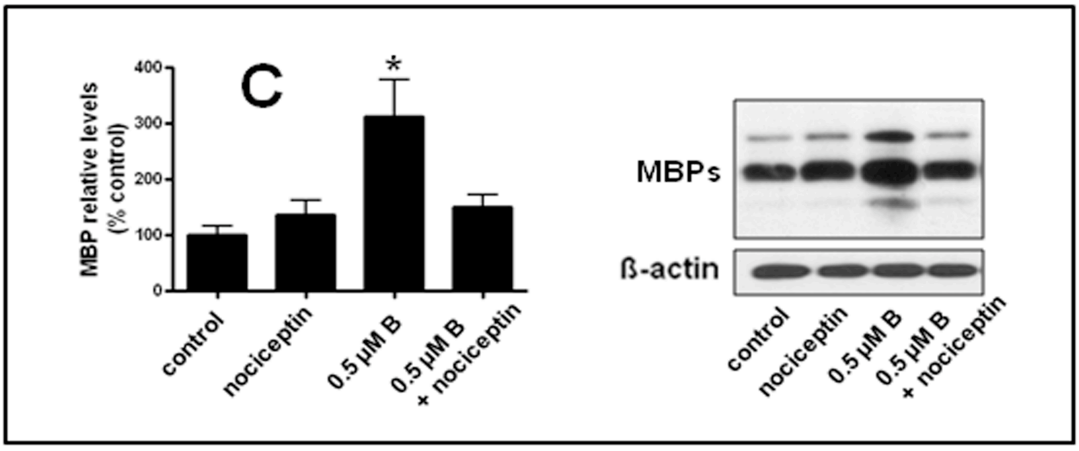
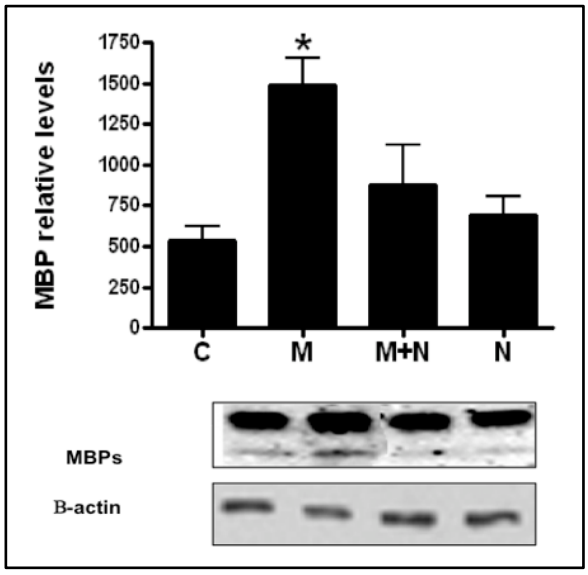

C
*
MBP relative levels
(% control)
400
300
200
100
0
control
nociceptin
0.5 µM B
0.5 µM B
+ nociceptin
MBPs
ß-actin
control
nociceptin
0.5 µM B
0.5 µM B
+ nociceptin

Figure 17: Specific receptor activation with methadone and nociceptin replicates MBP expression with low and high doses of buprenorphine. Cells were cultured in the presence or absence of methadone (M) with or without 1 μM nociceptin (M+ N and N). Results are expressed as percentage of controls ± SEM from four experiments and correspond to the combined scanning of the four major MBP isoforms. *P <0.05. MBP levels were determined by western blotting using ß-actin levels as loading controls.


MBP relative levels
1750
1500
1250
1000
750
500
250
0
*
C
M
M+N
N
MBPs
B-actin

MBP expression and myelination in the developing brain are also affected by methadone

The above results indicated that buprenorphine could directly alter OLG development through a novel interplay of opposing actions mediated by MOR and NOPR. Furthermore, these *in vitro* effects mirror the dose-dependent actions of buprenorphine previously observed in the *in vivo* studies (Sanchez et al., 2008). Interestingly, these findings also revealed that, at least *in vitro*, OLG development is stimulated by activation of MOR by methadone. This is particularly important and intriguing because as discussed before, methadone is the FDA approved drug for the treatment of pregnant opioid addicts.

Thus, we next sought to determine whether similar effects of methadone would also be observed in an *in vivo* model. For this, pregnant rats were implanted with osmotic minipumps to deliver water (controls) or methadone at a dose of 9 mg/kg/day, which is comparable to that administered to pregnant women. By using this experimental paradigm the pups were first exposed to the drug through the placenta and then through the maternal milk until the time of sacrifice at 11 and 19 days of age.

Similar to the results observed with the cultured cells, analysis of the pup brains at both 11 and 19 days of age indicated that *in vivo* exposure to methadone also results in a significant increase in MBP expression (**Figure 18**).

Furthermore, preliminary studies in which the brain tissue was visualized by microscopy after staining with toluidine blue, point to the possibility that the elevation of MBP levels in

methadone-exposed pups correlates with increased myelination. Images from the corpus callosum of 19-day-old animals, suggest a relative increase in the total number of myelinated axons in the pups exposed to methadone **(Figure 19)**. In addition, a greater percentage of these myelinated axons appear to exhibit thicker myelin sheaths in the methadone-treated animals than in the control. Studies are already in progress to quantitate these results and to employ electron microscopy for an in depth analysis of potential differences in myelin structure and thickness.

Altogether, these observations further demonstrate the role of opioids in myelination and the need to further study how exogenous opioids could affect brain development.

Figure 18: Increased brain MBP expression *in vivo* due to methadone exposure through the placenta and maternal milk. Pregnant rats were implanted with minipumps releasing either water for control pups or 9 mg/kg/day methadone. At 11 **(A)** and 19 days **(B)** of age, MBP brain expression was measured by western blot analysis. The figure shows representative western blots. **P<0.01, ***P<0.001.

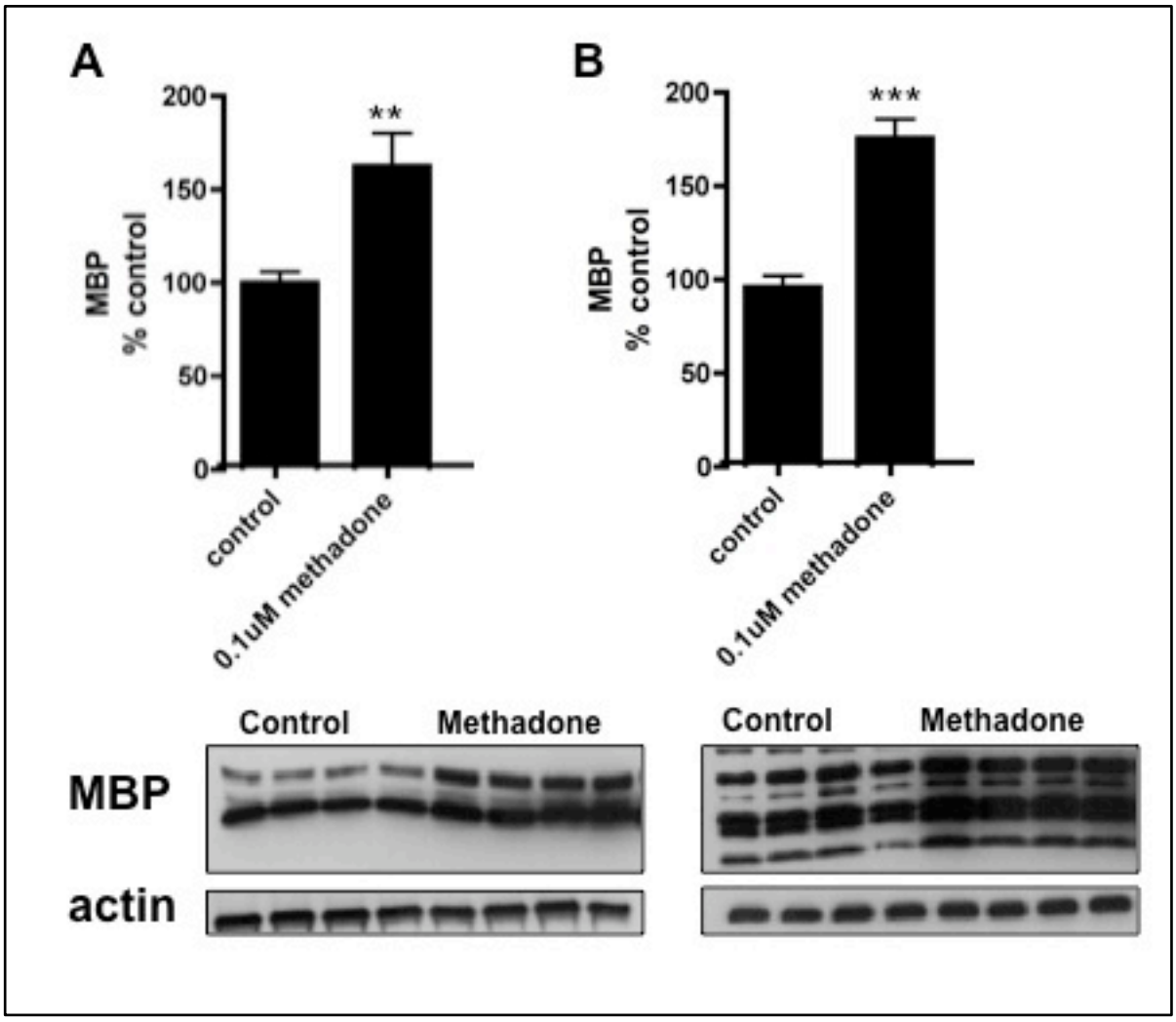
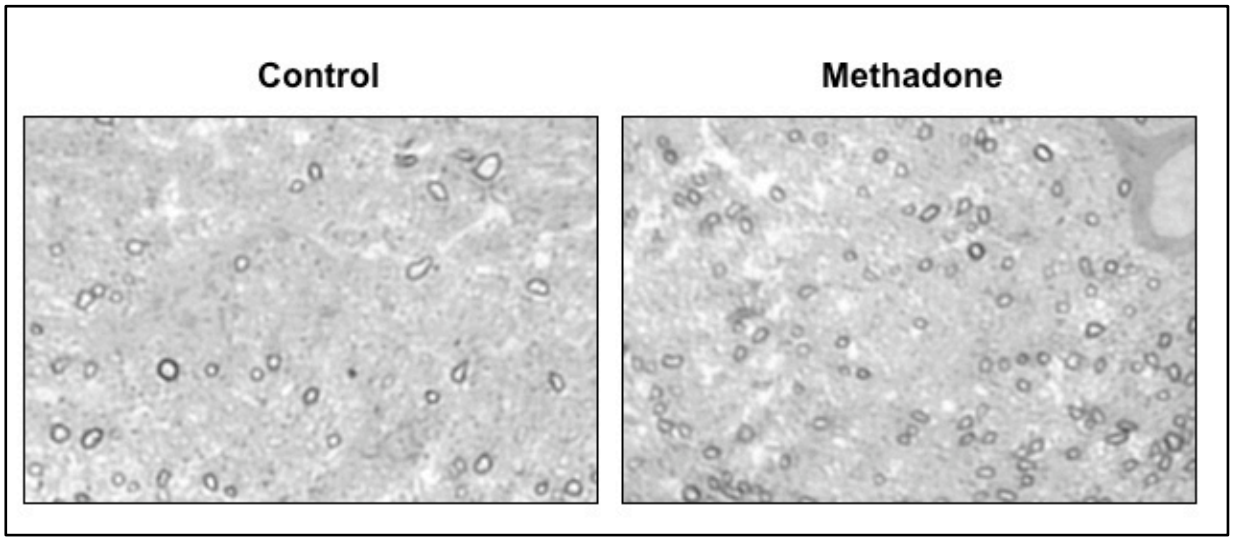

A
200
150
100
50
0
MBP
% control
**
control
0.1uM methadone
B
200
150
100
50
0
MBP
% control
***
control
0.1uM methadone
Control
Methadone
Control
Methadone
MBP
actin

Figure 19: Representative microscopy images of brains from control pups and pups exposed to 0.1 μM methadone *in vivo*. Pregnant rats were implanted with minipumps releasing either water for control pups or 9 mg/kg/day methadone. At 19 days of age, the brains of these pups were isolated and fixed and slices of the corpus callosum were stained for myelin. Representative images for control and methadone animals are shown.


Control
Methadone

DISCUSSION

Previous observations from this laboratory demonstrated that rat brain myelination is altered by buprenorphine (Sanchez et al, 2008), an opioid analogue currently used in clinical trials for the treatment of pregnant addicts. The studies in this thesis now showed that OLG development and myelination could also be affected by methadone, which unlike buprenorphine is the already FDA-approved treatment for opioid addiction during pregnancy. These studies also led to the finding that balance between opposing effects mediated by MOR and NOPR signaling play a crucial role in regulating OLG maturation and may therefore affect the timing of myelin formation. Furthermore, exogenous opioids like buprenorphine and methadone may disrupt this normal interplay, altering the developmental pattern of brain myelination. These findings are particularly interesting when considering the rising trend of opioid abuse.

The scale of the international problem of opioid dependence is increasing, with a current estimate of nearly 16 million users of illicit opioids worldwide (Health, 2009). In addition to the clear health concerns, drug abuse represents a great economic burden in industrialized countries, with opioid abuse accounting for roughly 2% of the gross domestic product (Health, 2009). In 2002, the total cost of drug abuse in the United States was estimated to be $180.8 billion (Strassels, 2009). Opioid abusers also require more resources and were found to be more likely to visit emergency rooms, having both more hospital stays and outpatient visits than non-abusers (White et al., 2005). In recent years, the increase in opioid abuse reflects a much larger population of over 2 million new abusers using pharmaceutical opioids in contrast to roughly 90,000 new users of illicit opioids such as heroin (Substance, 2009).

However, despite the unfortunate amount of abuse and dependence, this class of drugs has clear medical benefits and the past decade has been characterized by a significant increase in opioid administration for pain control in adolescents and young adults (Thomas et al., 2006, Fortuna et al., 2010). In the United States, this demographic group was prescribed with a controlled medication in up to 16% of ambulatory care interventions, with a third of these cases being related to back/musculoskeletal pain or injury (Fortuna et al., 2010). This specific age group also exhibits a similar increase in the prevalence of physical pain conditions relating to puberty and development (LeResche et al., 2005) and this may play a role in the augmented use of opioids.

Therapeutic opioids are also prescribed as part of treatments for the management of opioid abusers, assisting with withdrawal symptoms and improvement of patient outcomes. Among these medications are methadone and buprenorphine, two opioid agonists that have been shown to greatly reduce additional opioid use in addict patients (Bolnick and Rayburn, 2003, Johnson et al., 2003, Amass et al., 2004, Lacroix et al., 2011). Heroin-dependent adult inmates that received both counseling and treatment with methadone while still in prison were significantly more likely to stay in treatment and less likely to test positive for opioids 6 months after release than those subjected to counseling alone (Gordon et al., 2008).

As discussed before, similar therapies with methadone or buprenorphine are also currently used for pregnant women, both in their treatment of opioid addiction as well as in pain management. As previously described, these drugs can pass through the placenta, possibly

altering the development of the fetus. Furthermore, despite the additional exposure of infants to these drugs through breast milk (Begg et al., 2001, Jansson et al., 2007), some physicians continue to promote breastfeeding by mothers who use opioids (McQueen et al., 2011). Approximately half of these infants exposed to opioid maintenance therapies will require medical treatment after birth due to their own neonatal abstinence syndrome (Jones et al., 2010). However, the general outcomes of these medication therapies are positive. Infants from mothers in these therapy programs had improved birth weights and a greater percentage remained in maternal care one year after birth (Meyer et al., 2012). Additionally, these treatments appear to be generally safe for the infant, with no significant difference in mortality rates between children with and without methadone exposure (Kelly et al., 2012). However, while these treatments improve the outcome of pregnancies of opioid abusers, it is important to fully understand how these drugs may be affecting both the developing fetus through the placenta and also infants through breast milk. Interestingly, a Norwegian study found that children whose mothers were prescribed opioids more than 15 times between 2004 and 2009, were themselves also more likely to be prescribed opioids more than 4 times during that same period (Log et al., 2012). While this study cannot answer whether this increase in opioid use among children is truly an effect of earlier opioid use of the mother, it raises an interesting relationship of multigenerational drug patterns.

Opioid use causes alterations in GPCRs, second messengers and enzymes, which can mediate the actions of opioids and reinforce continued use (Nestler et al., 1993, Nestler, 1997). For example, MOR agonists, such as morphine and methadone, have been found to increase dopamine release from the nucleus accumbens and dorsal caudate, while KOR agonists

decreased the release of dopamine (Di Chiara and Imperato, 1988). Additionally, perinatal exposure to methadone was shown to disrupt cholinergic development in rats, as measured by an increase in acetylcholine turnover (Robinson et al., 1996). Furthermore, early studies implicated MOR as a stimulator of proliferation for both neonatal OLG progenitors (Knapp and Hauser, 1996) and adult neuroprogenitors (Persson et al., 2003).

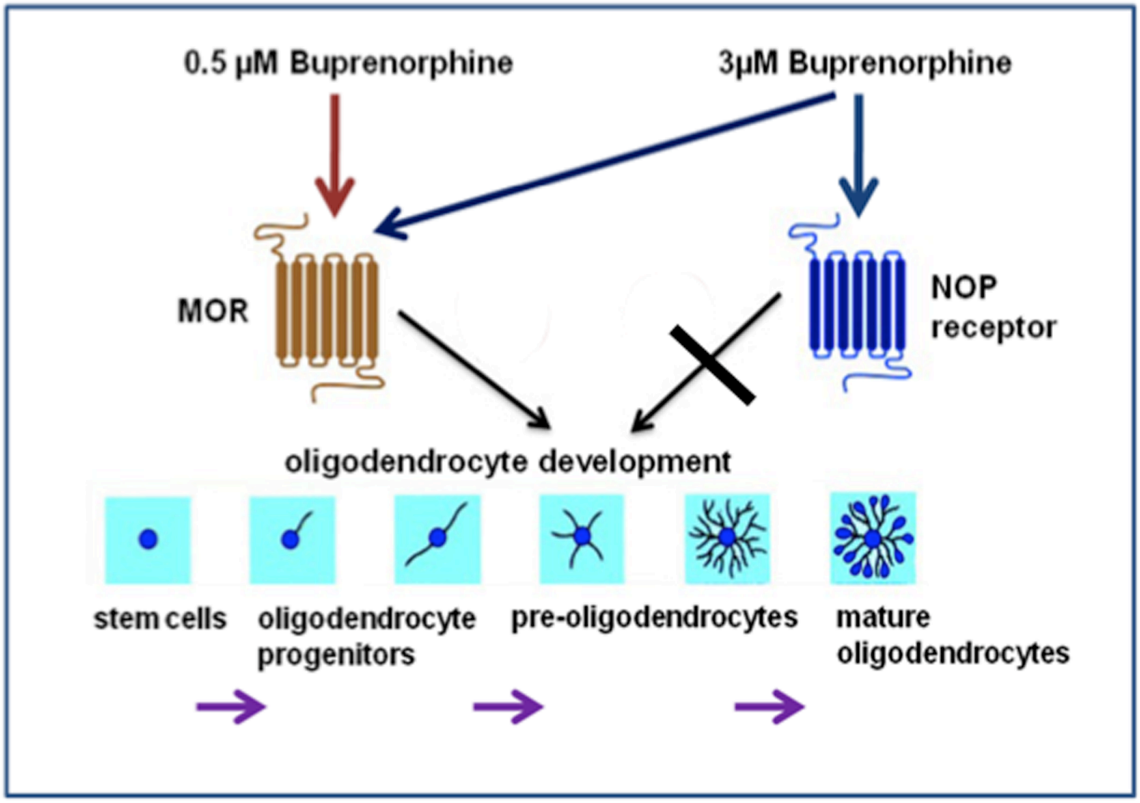
This laboratory has previously demonstrated that perinatal exposure to buprenorphine had dose-specific effects on myelination in the rat brain (Sanchez et al., 2008) and that low doses of buprenorphine exerted their effects through MOR to result in an increase in MBP expression and increased morphological complexity of the OLGs (Eschenroeder et al., 2012). This thesis further investigated the mechanisms underlying buprenorphine effects on OLG maturation and on developmental myelination.

Prior studies on buprenorphine described this synthetic opioid as a partial agonist of MOR (Cowan et al., 1977, Clark et al., 2006), antagonist of KOR (Leander, 1987) and both agonist and antagonist of DOR (Huang et al., 2001). This thesis describes novel findings indicating that, not only do OLGs express NOPR (**Figure 13**) but together with MOR, this receptor is also responsible for the observed bell-shaped dose-response effects of buprenorphine on these cells. Interestingly, high doses of buprenorphine work through NOPR **(Figure 14)** opposing the stimulatory actions of the drug which we found to be mediated by MOR, resulting in increased MBP expression and OLG differentiation (**Figure 10** and **Figure 11**).

Our findings are consistent with a model in which buprenorphine exerts direct effects in the OLGs by binding to two different receptors with opposing roles on cell development **(Figure 20)**. Because the affinity of buprenorphine for NOPR is lower than its affinity for MOR (Toll et al., 1998), it is logical to suppose that low drug levels activate the high affinity MOR while high buprenorphine concentrations are required to bind to both MOR and the low affinity NOPR. MOR activation results in stimulation of OLG maturation while this effect is counteracted by an inhibitory effect induced by concomitant signaling through NOPR. Therefore, it is tempting to hypothesize that balance between these two pathways may play a crucial role in "timing" OLG differentiation and the beginning of myelin synthesis. Buprenorphine, methadone and other opioid drugs could alter this interplay affecting myelination in the maturing brain.

Our preliminary studies aimed at understanding the mechanism behind the increase in MBP expression induced by MOR, suggest that the elevation of protein levels induced by methadone is preceded by a similar increase in the activity of the MBP gene promoter (data not shown). Importantly, these MOR-dependent effects are not limited to cultured OLGs as they are mirrored by an increase in MBP expression and myelination in the brain of rat pups perinatally exposed to methadone through the placenta and maternal milk (**Figure 18** and **19**). Therefore, future work in this area may use this as the starting point to investigate the connection between MOR activation and increased OLG maturation. Based on our previous *in vivo* (Sanchez et al., 2008) and *in vitro* results (Eschenroeder et al., 2012) thesis), it is tempting to hypothesize that the MBP gene is also subjected to regulation by NOPR, limiting expression of the protein until the beginning of myelination.

Figure 20: The dose-specific effects of buprenorphine in oligodendrocyte development. Low concentrations of buprenorphine activate the high affinity MOR, while higher doses of buprenorphine additionally bind NOPR with concurrent MOR activation. Sole MOR activation results in a stimulation of OLG development, an effect that is counteracted by inhibition due to signaling through NOPR. The balance between these receptor activities may modulate oligodendrocyte differentiation. (Eschenroeder et al., 2012)


0.5 µM Buprenorphine
3µM Buprenorphine
MOR
NOP
receptor
oligodendrocyte development
stem cells
oligodendrocyte
progenitors
pre-oligodendrocytes
mature
oligodendrocytes

Elevation of MBP levels and perhaps MBP gene activation induced by MOR may involve different signal transduction mechanisms. The MOR agonist, morphine, has been shown to activate ERK (Rothe et al., 2012) and this protein kinase has been previously shown to regulate OLG differentiation (Fyffe-Maricich et al., 2011), survival (Althaus and Kloppner, 2006) and myelination (Haines et al., 2010). Loss of ERK2 resulted in fewer mature OLGs and therefore, decreased MBP levels. In addition, treatment of cultured OLGs with an inhibitor of MEK (the kinase that activates ERK) also resulted in a greater percentage of cells with immature morphologies and suggested a role of an ERK-dependent pathway on cell process formation and branching (Younes-Rapozo et al., 2009). One potential downstream target of MOR and ERK is Sp1, as phosphorylation of this transcription factor is known to be regulated by ERK-mediated pathways (Guo et al., 2010).

Importantly, Sp1 was previously shown to activate the MBP gene (Wei et al., 2003a) through binding to a GC-rich region of the promoter (Tamura et al., 1989, Tretiakova et al., 1999). Interestingly, Sp1 was found to mediate the upregulation of MBP gene activity induced by elevated levels of p27(Kip1) (Wei et al., 2004), a cyclin-dependent kinase inhibitor whose expression is increased with terminal differentiation of OLGs (Casaccia-Bonnefil et al., 1997, Durand et al., 1997). This mechanism involves a p27(Kp1)-dependent stabilization of Sp1 (Wei et al., 2003b)

Sp1 may also play a role in controlling the levels of Fyn, a Src-family tyrosine kinase that has been implicated in OLG development (Umemori et al., 1994, Osterhout et al., 1999). Experiments in chronic myelogenous leukemia cells showed that Sp1 mediates the up-regulation

of Fyn expression that occurs in response to oxidative stress (Gao et al., 2009). This potential role of Sp1 is particularly interesting because early studies showed that Fyn could regulate MBP expression by a downstream mechanism that ultimately causes MBP gene activation (Umemori et al., 1999). More recently, the Fyn kinase was shown to phosphorylate the heterogeneous nuclear ribonucleoprotein F (hnRNP F), which results in its release from MBP mRNA, allowing translation (Laursen et al., 2011, White et al., 2012). In this regard, previous results from this laboratory showed that *in vivo* exposure to low doses of buprenorphine, which led to a significant increase in MBP expression, also resulted in increased interaction of Fyn with MAG (Sanchez et al., 2008), a glycoprotein that may play a crucial role in the early stages of myelination and axo-glial interactions (Quarles, 2007). However, total brain levels of Fyn in those animals were normal and higher complex formation with MAG was suggested to perhaps be dependent on the observed increase in MAG glycosylation. Nevertheless, it remains to be determined if Fyn levels could be specifically altered in the OLGs by a MOR-dependent mechanism and whether Sp1 may be involved in this or any of the other mechanisms discussed above. However, activation of NOPR by its endogenous peptide nociceptin has also been found to stimulate the ERK pathway (Lou et al., 1998) and could therefore lead to MBP promoter activation through Sp1 phosphorylation, a situation that would contradict our findings of opposing roles of MOR and NOPR on MBP expression.

Another potential mediator of MOR effects in OLGs and myelination could be the mammalian target of rapamycin (mTOR), because at least in human non-small cell lung cancer, over-expression of MOR resulted in mTOR activation (Lennon et al., 2012). Results from this and other laboratories have shown that mTOR appears to play a role in the promotion of OLG

differentiation (Coelho et al., 2009, Tyler et al., 2009, Guardiola-Diaz et al., 2012). Akt activates mTOR and it has been previously shown that constitutively active Akt results in hypermyelination, without an increase in the number of OLGs present in the CNS (Flores et al., 2008). Results from our laboratory suggested that the mTOR signaling pathway stimulates protein expression in the developing OLGs by targeting both the eukaryotic initiation factor 4E (eIF4E), and its inhibitory binding partner 4E binding protein 1 (4EBP1), two very crucial players in the control of cap-dependent protein synthesis (Coelho et al., 2009). Therefore, it is possible that methadone and low doses of buprenorphine, working on MOR, may increase mTOR activation and lead to increased myelination after OLG differentiation.

It remains to be investigated how NOPR counteracts the positive effects on OLG development induced by MOR. The endogenous NOPR ligand nociceptin has also been found to stimulate ERK (Lou et al., 1998) and NOPR has additionally been shown to also activate ERK in the fluid percussion brain injury model (Ross and Armstead, 2005) and in the nucleus accumbens (Chen et al., 2008). However, NOPR is also known to inhibit adenylate cyclase (Meis, 2003), an enzyme involved in the activation of CREB, a transcription factor for which results from this and other laboratories demonstrated a role as a mediator of multiple signals that modulate the extent of OLG development and myelination (Sato-Bigbee et al., 1999, Afshari et al., 2001, Saini et al., 2004, Saini et al., 2005, Bhat et al., 2007). Furthermore, acute morphine treatment is known to result in increased CREB phosphorylation (Wang and Burns, 2009) and thus, it is possible to hypothesize that antagonistic effects of MOR and NOPR may be in part mediated by their reciprocal effects on CREB activation.

The role of opioids in the regulation of myelination is not only of interest during development, but also in disease states such as MS. Due to neuropathic pain associated with MS, some patients are prescribed opioids for their anti-nociceptive properties (Solaro and Messmer Uccelli, 2010). This is important in light of our results presented in this thesis, as the treatment given to the MS patient for pain may also be playing a role in the progression of MS by affecting the differentiation of progenitor cells into mature myelinating OLGs through binding to the opioid receptors. Actions on NOPR may have downstream effects that inhibit the re-myelination of affected CNS areas. Intriguingly, in preliminary, yet to be published, results from this laboratory, nociceptin was found to be up-regulated in CNS tissues of MS patients (data not shown). Therefore, in addition to possible effects on OLG differentiation due to exogenous opioids given for pain, increase of nociceptin could work against re-myelination of the CNS through NOPR activation.

Furthermore, opioids have also been implicated in the modulation of symptoms in experimental allergic encephalomyelitis (EAE), an animal model of MS. Treatment with the opioid peptide Met(5)-enkephalin resulted in decreased exhibition of the clinical signs of EAE and an increased rate of remission after one month of treatment (Zagon et al., 2010). Additionally, this same laboratory found that long-term treatment with this opioid continued to stop disease progression and had no harmful long-term effects at two months of treatment (Rahn et al., 2011). Furthermore, within 10 days, there were fewer activated astrocytes measured in the groups treated with the opioid (Rahn et al., 2011). Previous studies have found that reactive astrocytes may produce ceramide, which then induces the migration of monocytes, furthering the pathology of MS (van Doorn et al., 2012). Therefore, it may be that the treatment with

Met(5)-enkephalin improves the remission of EAE, in part, through a reduction of reactive astrocytes.

The importance of understanding how exogenous opioids could affect the OLGs has been recently shown through the implication of OLG pathologies in bipolar disorder (McIntosh et al., 2009, Mahon et al., 2010), major depressive disorder (Hamidi et al., 2004, Aston et al., 2005) and schizophrenia (Haroutunian et al., 2007, Segal et al., 2007). Interestingly, examination of postmortem brains of schizophrenics (Hakak et al., 2001, Pongrac et al., 2002) and bipolar disorder (Tkachev et al., 2003) patients showed decreased expression of PLP and other myelin-related genes.

The effects of exogenous opioids on the CNS have been studied in opioid abuse patients, a population found to have deficits in the areas of attention, recall and concentration (Gruber et al., 2007) and in the structure of the white matter (Lyoo et al., 2004, Bora et al., 2012). Interestingly, one study found that these deficits in executive and memory function associated with chronic drug users were not associated with the length of time of either abuse or abstinence post-abuse (Ersche et al., 2006). Further work in this area has demonstrated that, regardless of whether methadone or buprenorphine was used, the length of maintenance treatment was positively associated with a poorer performance in tests measuring sustained attention (Loeber et al., 2012).

However, opioids are commonly not the only drug type in abuse patients and these other abused drugs may also affect OLGs and myelination. Cocaine is a powerful stimulant derived

from the *Erythroxylum coca* plant and works by either blocking the re-uptake of serotonin, dopamine and noradrenalin or by blocking voltage-dependent sodium channels (Ritz et al., 1990). Polydrug use of cocaine by patients in methadone or buprenorphine maintenance treatment has been found to be as high as 73% (Magura et al., 1998). The amount of cocaine abuse in opioid users not in treatment was found to be even higher, reaching 92% (Hasin et al., 1988). Additionally, rats treated with cocaine have been found to exhibit cross-sensitization to the conditioned rewarding effects of morphine (Shippenberg et al., 1998) and in a similar fashion, morphine treated rats exhibited cross-sensitization to the locomotor effects of cocaine (Cunningham et al., 1997). A further study of this phenomenon found that while agonist activity of KOR negatively modulates this cross-sensitization, agonists for MOR positively modulate this effect between opioids and cocaine (Smith et al., 2009).

Interestingly, studies of human cocaine abusers have identified a loss of mature, MBP positive OLGs (Bannon et al., 2005). In agreement with this observed effect of cocaine on mature OLGs, work with EAE rats has shown that cocaine exposure caused a delay in the improvement of neurological signs, as did amphetamines, which was suggested to be related to the increase in immune cell migration (Nunez et al., 2007). Other work has shown that cerebral vasoconstriction as a result of the cocaine (Kaufman et al., 1998) can be linked to hypoperfusion, which in turn has been shown to decrease MBP (Kurumatani et al., 1998). However, the deleterious actions may also be due to the previously documented inhibitory effects on OLGs and the process of re-myelination. Furthermore, the levels of PLP expression have also been shown to be decreased in a subpopulation of human cocaine abusers (Lehrmann et al., 2003).

In summary, the results of this thesis showed that exposure to the exogenous opioids buprenorphine and methadone can significantly alter OLG development, and they do so by a novel and complex mechanism that involves a delicate balance between MOR and NOPR mediated pathways. It is tempting to hypothesize that endogenous opioids and nociceptin may be part of a system by which neurons and astrocytes could signal to OLGs to myelinate the axons during development or in disease states. Exposure to exogenous opioids through treatment or abuse could affect this system, altering OLG differentiation and the process of myelination. Thus, further work is needed to understand the molecular players of these mechanisms, a problem which is especially important for the design of treatments to effectively manage opioid addiction in pregnant women and adolescence, minimizing potential effects at important developmental stages of rapid myelination.

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Publications

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